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PERIPHERAL NEUROGENIC TUMOR*

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It is the purpose of this paper to set forth as practically as possible the salient features in diagnosing tumors of the peripheral nerves, including those of the sympathetic system as well. The subject matter will deal chiefly with the microscopic appearance of sections from such tumors, as their gross appearance gives very little clue as to their true identity. It must be emphasized at the outset that it is quite futile to attempt to make accurate diagnoses of nerve tumors from sections stained with the routine hematoxylin-eosin, or eosin-methylen blue technics; it is only by using methods that stain or impregnate nerves and their adnexas differentially that one may hope to arrive at conclusions that will approximate accuracy. This may be done by employing such methods as the Masson trichrome aniline blue, or lichtgrün stain[3], or various silver impregnations; it is always well to use several methods and combine the impression thus obtained before trying to draw conclusions from a given block of tissue.

Besides this necessity for differential stains, it is also of importance to know the histology of the organ from which the tumor under examination has arisen. This is particularly true of the peripheral nerve trunks and their specialized endings, in which there is a variety of tissues represented. To review this histology briefly one may consider the following points: The neurones emerge as elongated processes from nerve cells in the brain or spinal cord, around them is the myelin sheath and the sheath of Schwann. These may thus far be considered as ectodermal in origin. Then comes the delicate sheath of Henle, which is probably mesodermal. The sheath of Schwann was formerly con-

* Read before the Fourteenth Annual Convention of the American Society of Clinical Pathologists held at Atlantic City, June 7 to 9, 1935.

sidered to be derived from the mesoderm, but the ectodermal theory is growing in popularity although it is still disputed and although Harrison (quoted by Maximow and Bloom,[15]) performed an ingenious experiment that should prove conclusively that the cells of this envelope are derivatives of the ganglionic crest. He removed this crest from frog embryos and allowed them to go on developing; the posterior roots of the spinal cord failed utterly to appear, while the anterior roots developed as usual, excepting for the important fact that they quite lacked a sheath of Schwann. The surrounding mesodermal elements, then, did not supply this layer as they were formerly supposed to do. In spite of this experiment there are many well-known authorities who persist in maintaining the mesodermal origin of this sheath. Its histology need not be described in detail; it will suffice to mention that it invests the nerve fibers from their point of emergence from the spinal cord to their very ultimate ending in nerve-plates, sensory organs and the like. Thus, for example, the bulk of the tactile corpuscles of Meissner is probably composed of modified Schwann cells arranged transversely with delicate nerve filaments coursing around and among them to end in equally delicate clubbed extremities.

The tinctorial characteristics of the sheath of Schwann differ from those of the other investing membranes, as do its microchemical reactions: it stains like neuroglia in the trichrome processes, not like collagen; it does not swell in dilute acetic acid, as does collagen, thus differing from it materially. The origin of this sheath is the most important controversial point to be kept in mind in connection with the diagnosis of tumors of nerve trunks.

Undisputedly mesodermal elements are the epineurium, which surrounds the trunk as a whole, the perineurium that envelopes its component bundles and the endoneurium that penetrates these and surrounds still smaller groups of nerve fibers. As a given nerve trunk dwindles in size, these mesodermal elements become very thin until they resemble an endothelial, or mesothelial sheet and are merely one cell-layer thick. The epineurium disappears and the peri- and endo-neurium become fused to form an indistinguishable union that is now known as the "sheath of Henle,"

the "connective peritubular tunic" of Ramon y Cajal, or the "subsidiary sheath" of Ruffini. These mesodermal elements have all the characteristics of collagenous or reticular tissue; they may be beautifully demonstrated in silver impregnations, as has been shown by Laidlaw.[5] A very good discussion of the peripheral nerve trunks may be found in the excellent text-book of Maximow and Bloom, from which much of this review has been taken.

NON-MALIGNANT TUMORS OF NERVE TRUNKS

In discussing tumors of the peripheral nerves, one must follow the histological outline just reviewed and adhere to it strictly, in no other way can any progress be made.

Tumors of neurones

Pure tumors of neurones are very rare, I have seen but one in some twenty years of experience. These are the true neuromas and they are composed of coiled masses of axis cylinders of varying size, invested with their membranes. This investiture may be rather haphazard, so that the individual neurones, with their sheaths of Schwann, lie embedded in a matrix of mesodermal, fibrous tissue. Two kinds of neuroma are recognized: (a) the rare, true neuromas just referred to (which after all may merely be developmental defects similar to plexiform angiomas) and (b) the false, or traumatic neuromas that commonly arise at the severed ends of nerve trunks, particularly in the case of the sciatic nerve where "amputation neuromas" are the bugbear of surgeons. They are scarcely to be considered tumors. A possibly similar overgrowth of the nervous elements in the appendix, following appendicitis or presumably connected with fibrous involution of the organ, is the "appendiceal neuroma" of Masson,[10,11] which represents a proliferation of the nerves composing the plexuses of Auerbach and Meissner and is frequently found in connection with obliteration of the lumen. The amputation neuroma is a clubbed overgrowth of coiled trunks at the extremity of the severed nerve that may attain considerable proportions; in the appendiceal neuroma one sees nerve trunks with a liberal supply of accompanying ganglion cells growing into the fibrous submucosa

from the muscular layers and forming a retiform mass of mixed nerve and smooth muscle (neuromuscular complexes).

Tumors of the epi-, peri- and endo-neurium

Growths of these sheaths are not difficult to diagnose as they are purely collagenous and hence true fibromas. They may follow injury to a nerve as a result of the organization of a small hematoma, or they may occur spontaneously; they are of more importance in the latter instance, but even so their chief interest lies in their possible connection with "neurogenic sarcomas" one type of which they may produce if they undergo malignant transformation. A non-malignant tumor of this sort would be a true "perineural fibroma"; this term is also used, probably erroneously, for lemmonas.

Neurinoma or schwannoma

Tumors arising from the sheath of Schwann are a subject of controversy, even as the origin of the sheath is disputed. They are known by an annoying array of aliases: schwannoma, neurinoma, lemmoma, perineural fibroma and even neuroma, when found in the sheath of the acoustic nerve (fig. 1*). The term Schwannoma was coined by Masson who studied these growths extensively and intensively; he admits the ineptness of the name, but states in personal correspondence, that he could not advocate the term "peripheral glioma" (which is excellent) because it had been already used in another sense. "Schwannoma" is not only ugly, but it combines a patronymic with a Greek ending meaning "tumor" and is therefore illogical and a distinct hybrid. Lemmoma is a much better designation, but it has not gained the favor that it should. If, on the other hand, one believes that the sheath of Schwann is just so much mesodermal tissue "perineural fibroma" would be quite acceptable. These tumors have been experimentally produced by Nageotte and Guyon and studied by Masson.[13] A segment of the sciatic nerve of one leg was

* All photomicrographs were taken at a magnification of x 320 by Miss E. Dreyfoos of the Department of Photography of the Cornell University Medical College with the assistance of the author.

removed from a rabbit and sewn into place inside the epineurium of the sciatic of the opposite side, in close approximation to the intact trunk. Tumors developed at either end of the implant, the cells composing them having all the characteristics of Schwann cells, rather than those of ordinary fibroblasts. The resulting growths were very similar to spontaneous Schwannomas.

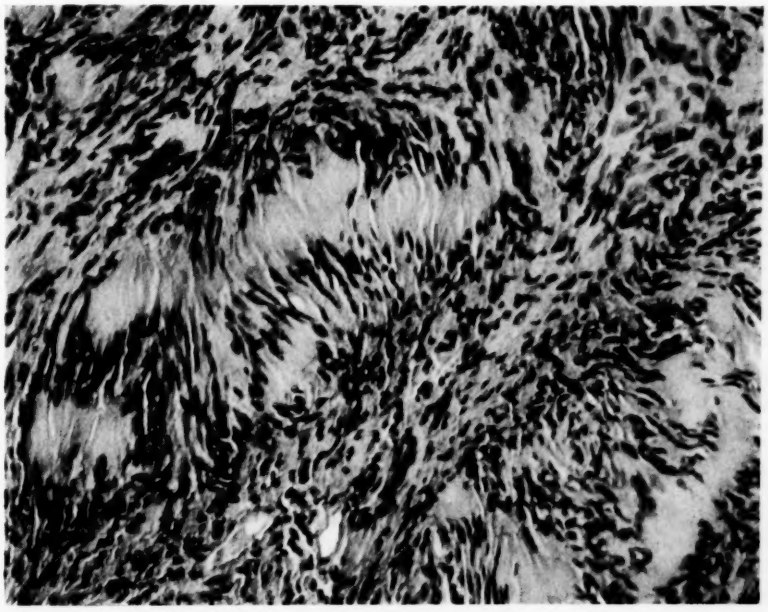

FIG. 1. BENIGN TUMOR OF PURE SCHWANN CELL COMPOSITION. Called Schwannoma, neurinoma, lemmona, et cetera. Notice the palisading of the nuclei with coarse, hyalinized fibers running between the palisades. When less regularly arranged and more whorled, they are known as Verocay bodies. (Masson hematoxylin-phloxin-saffron stain.)

The typical neurinoma is nodular, often elongated and arises along the course of a nerve, sometimes projecting from it in a sausage-shaped or spheroidal mass. It may surround the nerve-trunk, or lie along it and it may attain considerable size. Many of the "hour-glass tumors" of the spinal foramina are of this type. Microscopically two types of neurinoma are recognized: the A-type, or neurinoma proper and the B- or Antoni-type that shows

considerable mucoid degeneration and may be mistaken for a myxoma. The former is composed of elongated cells that are usually fusiform and may be excessively vacuolated, so that the nucleus lies in a foam-work of cytoplasm. They are arranged in fascicles and their nuclei are grouped side-by-side so that they form palisades or rows like soldiers. There may be large, concentric whorls of cells similar to those seen in meningiomas and in pedunculated moles of the scalp. In the neurinoma they are known as "Verocay bodies," after their discoverer. Masson holds them to be proof of the relationship of these tumors, one to the other. He traces their development from distorted Meissner corpuscles. Between the palisaded nuclei one finds parallel, coarse fibers that appear to be the tails of bipolar or fusiform cells and these are often inserted into hyalin central plates that stain like collagen and thus throw doubt on the ectodermal origin of the tumor. They resemble tendinous incisures in a muscle. As there is always a certain amount of overgrowth of the collagenous tissue in these tumors, it would not be stretching the point to assume that one element having become neoplastic, the others might share in the process, just as in the case of intracanalicular fibromas of the breast, for example. The Antoni, or B-type of neurinoma is explained by Masson on the basis that the collagenous elements of the tumor become hyalinized or "jellyfied" and spread out in such a way that they no longer appear like fibers. In such tumors the Schwann cells appear as fusiform, or anastomozing cells that might easily be mistaken for fibroblasts or reticulum cells and often are. The hyalin process extends to the vessels of the tumor as well in such instances and the general appearance is that of cellular myxoma.

Myxoma

There are myxomas of the peripheral nerves, both of the type just mentioned and those that arise from the perineural fibromas by myxomatous metamorphosis of the collagen they contain. The process is analogous to that occurring in any fibrous tumor or group of tumors.

Neurofibroma (von Recklinghausen's disease)

In this condition, which is quite familiar, there are tumors occurring along the course of nerves, intimately associated with them and composed of a variety of tissue. Apparently these neoplasms may be comprised of any, or all of the elements of the peripheral nerve trunk; they may be largely fibrous, or fibromas;

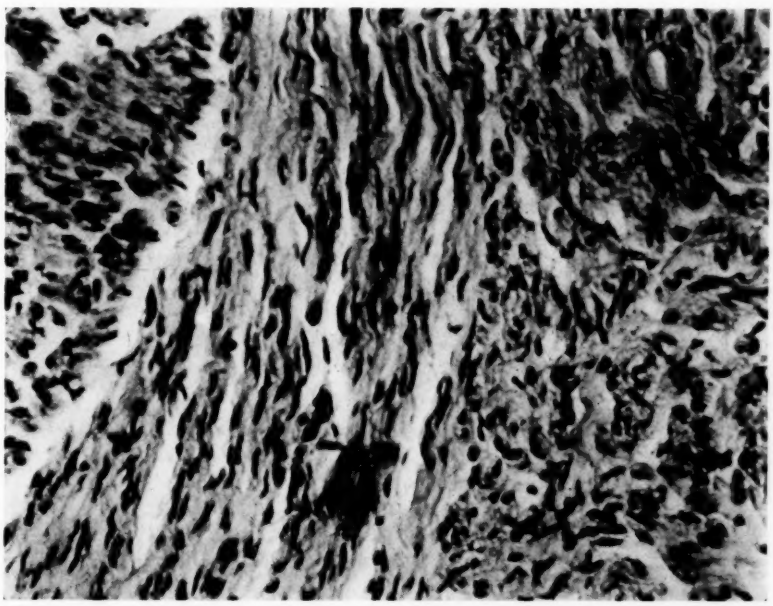

FIG. 2. BENIGN NEUROFIBROMA. Composed chiefly of the fibrous elements of the nerve sheath; the structures that simulate nerve trunks in this tumor seldom show actual axis cylinders in the rather well-formed sheaths. Schwann cells and fibroblasts share in the formation of the tumor. (Hematoxylin-eosin).

they may show actual overgrowth of nerve fibers, resembling true neuromas; they more usually show abortive trunks that contain most of the elements of these, excepting the neurones (fig. 2). Often there is great overgrowth of the Schwann cells, simulating neurinomatous growth. In the typical case there are multiple tumors appearing in the skin, or under it, anywhere in

the body; one may never be sure that each tumor will resemble its fellows, some of them may be simple fibromas, myxomas, or the mixture just alluded to. Furthermore, they may frequently contain melanoblasts or melanophores, a point that Masson has stressed to support his theory of the neurogenic origin of pigmented nevi. The traumatic neuromas, already described, may show such overgrowth of the sheath of Schwann and the fibrous sheaths, so as to resemble neurofibroma very closely.

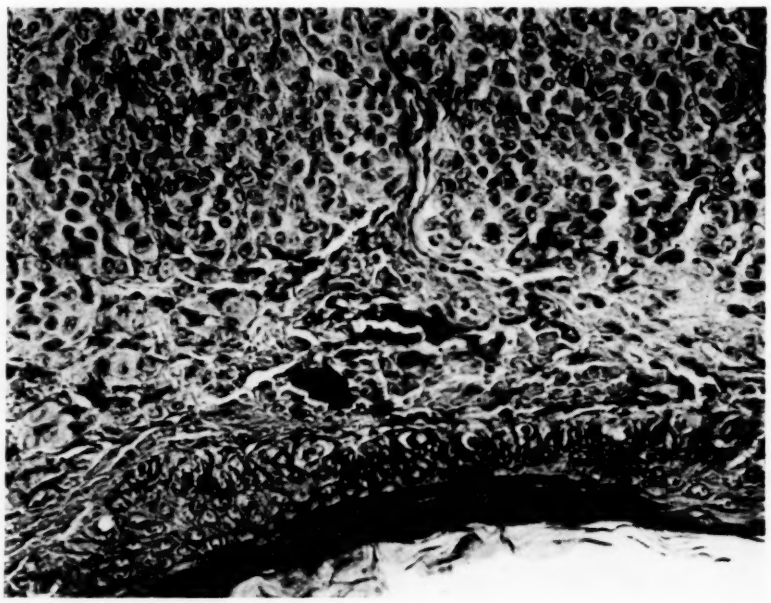

FIG. 3. BENIGN PIGMENTED NERVUS, OR MELANOMA, OF THE SKIN. Note the arrangement of the type cells (derived from the modified Schwann cells of the tactile corpuscles according to Masson) in compact nests. The melanin is concentrated in the more superficial parts of the tumor. (Masson trichrome lichtgrün.)

Pigmented nevi

At the end of the nerves, the terminal apparatus may also be the site of tumors (figs. 3 and 4). Tumors of the motor plates have not, so far as I know, been described. Thoma, quoted by Ewing,[2] has described small, painful globular tumors of the skin

that he traced to Pacinian corpuscles; they are very rare. Tumors of the tactile organs of Meissner and of the supposedly temperature regulating glomic bodies are coming to be recognized for what they are. Masson[5] has shown that the ordinary pigmented nevus, or mole, is really a tumor of the Meissner corpuscles and, less frequently, of the Merkel-Ranvier cells and nodes

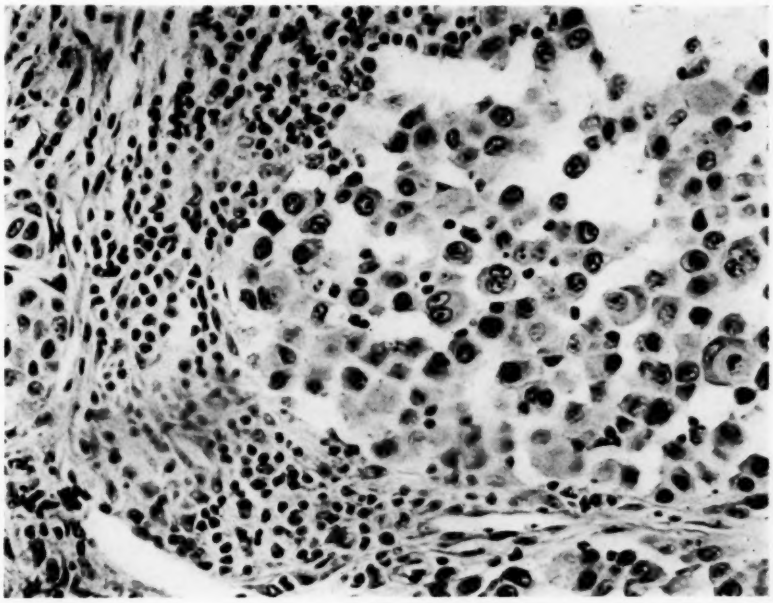

FIG. 4. A TUMOR QUITE SIMILAR TO FIG. 3 BUT UNDERGOING MALIGNANT CHANGE. The cell nests are less compact, the cells are larger and often multinucleated and mitotic figures are present. At the margin of the illustration are nests that still maintain the compact type of growth of the benign form of this tumor. (Masson trichrome lichtgrün.)

in the epidermis and that they are not derived from the epidermis as was formerly believed. So complete has been his proof and the confirmation of his work by other investigators, such as Laidlaw,[6,7] that so staunch a supporter of the epidermal theory of origin as Miesscher, of Zürich, has been won over completely to his view. These tumors are composed of specialized Schwann cells arranged in nests and closely resembling Meissner corpuscles

in some instances, while in other forms of pigmented nevi the analogy is not so striking, as the cells are arranged in simple alveoli, or nests. One can not hope to see such resemblances in hematoxylin-eosin, or VanGieson sections, it is only with Masson's methods or silver impregnations, that their real dissimilarity to the epidermis comes out strikingly. They may or may not contain melanin and Masson has come to the conclusion that the pigment is merely a matter of individual metabolism in these tumors and relatively unimportant. In rare instances one sees benign melanomas arising intraepidermally, the so-called "intracutaneous melanomas"; this is probably due to their developing from Merkel-Ranvier cells in the basal layer of the epidermis, intercalated between the basal cells, rather than from Meissner corpuscles which lie deeper in the papillae of the derma. They form large, flat and deeply pigmented spots that do not project above the skin level, nor penetrate deeply beneath it, lying entirely within the epidermis. Small forms of these are to be found on almost anyone, the pin-head pigmented spots that resemble dark freckles. In the scalp the melanomas are larger, more apt to be pedunculated and more frequently show distorted Meissner corpuscles, which Masson calls "lâmes foliacées." It was while studying these that he conceived his theory. These laminated bodies are similar to the Verocay bodies of neurinomas and the whorls of the meningiomas, they are sometimes seen in neurofibromas also. That they represent distorted Meissner corpuscles has been proved by the silver impregnations of Laidlaw,[6] who has demonstrated distorted nerve endings in their substance. He ascribes these tumors to legacies from our amphibian ancestry, as the pigmented spots on the snout of the alligator are tactile organs quite similar to Meissner corpuscles. These nerve-terminal tumors lie on the border-line between tumors and developmental defects, they are akin to hamartomas on the one hand and plain defects on the other, when one may find associated with them small angiomas, small leiomyomas, or small sebaceous or sweat-gland adenomas arising from the adnexas of the epidermis. These pigmented tumors may occur as vast areas of pigmentation, on occasions, such as the "bathing trunk nevi" whose name explains their distribution.

Tumors of the glomic bodies

It is questionable whether glomic tumors should be included in this paper or not; they are, to be sure, chiefly vascular, but as they are always associated with nerve terminals and filaments, so as to be called "vasculo-neuro-muscular complexes" it might be well to mention them for the sake of completeness. They usually occur beneath the finger-nails, where they form exquisitely tender and painful small nodules that gradually grow until they require surgical intervention. They must be differentiated from subungual melanomas and were not recognized until Masson took them under observation[9,14] after having spent considerable time on the histology of the glomus from which they arise. This is presumably a temperature-regulating organule composed of arterio-venous anastomosing coils of vessels that are liberally supplied with nerves and smooth muscle. Popoff[16] has recently written an extensive paper on their histology. The tumors are very rarely seen and, at present, as rarely recognized; any small tumor beneath the finger nail should at once arouse interest and suspicion and be investigated for the presence of distorted arterio-venous complexes associated with nerve and muscular tissue.

TUMORS OF THE SYMPATHETIC NERVOUS SYSTEM

Again one must bear in mind the histogenesis of sympathetic ganglia, the transition from the sympathogonia to the ganglion cell, the capsule cell, the supporting glial tissue and the pigmented phaeochromocyte. Most of the tumors of this group are malignant and will be described later. The best differentiated neoplasm is the ganglioneuroma (fig. 5), which may occur anywhere that sympathetic ganglia may be found. It shows groups of well-differentiated ganglion cells, surrounded by their capsule cells and embedded in a stroma of glial tissue; often it is so well-formed that it is difficult to decide whether one is dealing with a sympathetic ganglion or a tumor originating from one. It may grow to some size, but its gross appearance is not very distinctive.

Chromaffin tumors

The chromaffin cells are closely related to the nervous apparatus, they are argentaffin and show small, black granules when

properly impregnated with silver salts. They arise from cells, in the case of the intestines, that are intercalated between the epithelial elements of the crypts; in other organs they arise in situ, as in the suprarenal, carotid body, etc. In the former case they are known as "carcinoids, or argentafinnomas," in the latter as "paragangliomas." The carcinoids of the appendix look like carcinomas, with rather small cells and spherical, hyperchromatic nuclei; they are invasive, but they do not metastasize. Those

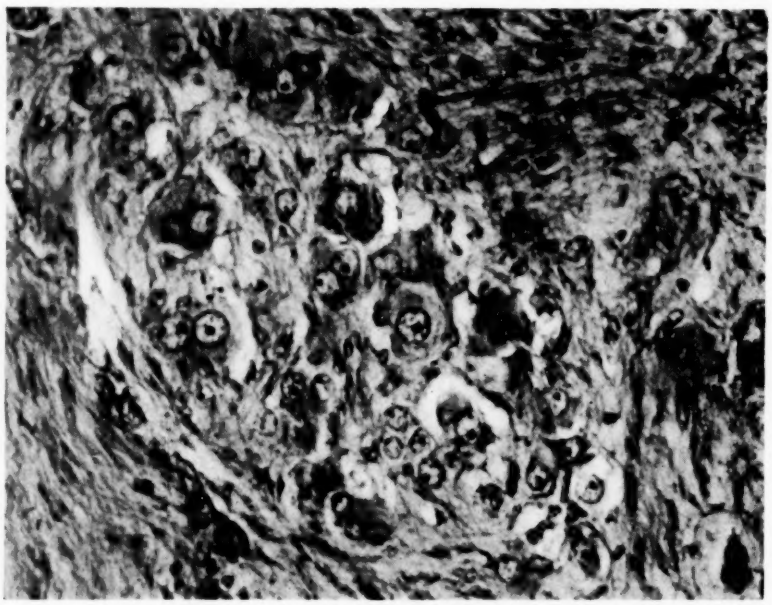

FIG. 5. BENIGN TUMOR OF SYMPATHETIC NERVOUS ORIGIN, THE GANGLIONEUROMA. Note the well-differentiated ganglion cells and the background of non-medullated nerve fibrils. (Masson hematoxylin-phloxin-saffron).

that arise in the small intestine, on the other hand, although they exactly resemble their appendiceal brethren, metastasize very frequently and extensively. Why this should be we do not know.

Carcinoids

In the appendix the carcinoid is a symptomless tumor, usually discovered at operation or at necropsy; it may escape detection until routine sections reveal its presence on microscopic examination. It causes a clubbing of the extremity of the organ, where

a bright yellowish mass is disclosed on section. It may be asymmetrical, lying at one side of the lumen, or it may surround it evenly. It is composed of small, cuboidal cells with small, spherical and hyperchromatic (rather stippled) nuclei growing in groups that resemble the alveoli of a medullary carcinoma, invading lymphatic channels and nerve sheaths and showing the distinctive affinity for silver already mentioned. Masson[12] has also investigated these tumors and believes that they grow out from the nerve cells at the base of the crypts which migrate into the submucosa and, according to him, invade nerves and spread out along them. The spread along nerve trunks is not wholly confirmed by the observations of other investigators. In the malignant form of the tumor that occurs in the intestine, there is no histological distinction at all, yet metastasis to the liver and ultimate death are the usual outcome. The clinical course of such cases, however, is usually somewhat slower than that of an ordinary adenocarcinoma.

Paragangliomas

Paragangliomas may be found in organs in close relation to the nervous system, such as the carotid body or suprarenal medulla. They resemble carcinoids in their general appearance in the carotid body and somewhat in their behavior. They may occur as simple adenomas, growing in cords of masses, or they may have a perivascular distribution, enclosing vessels in a sheath of tumor cells, in which case they are often known as "peritheliomas." Such tumors are very vascular and the surrounding cells flatter and more elongated than they are in the other type. Even when non-malignant they are very troublesome to the surgeon, as they so intimately surround the great vessels as to make their extirpation a very risky matter. They tend to recur after removal. Both forms may show malignant change and become "malignant paragangliomas."

MALIGNANT TUMORS OF PERIPHERAL NERVE ORIGIN

Neuromas

Neuromas may have a malignant form, but one never hears of them and I know of no reported cases.

Malignant neurinoma

Tumors of this type sometimes show malignant change and give rise to large tumors of nerve trunks that are often misdiagnosed and rarely recognized as such, they metastasize widely and several have been recently reported. They more or less resemble fibrosarcomas histologically and are often so diagnosed, they are extremely malignant and terminate fatally. They form one group of the "neurogenic sarcomas" and it is only with such stains as Masson's trichrome anilin blue or lichtgrün that one may hope to distinguish them from the fibrogenic form of neurogenic sarcoma. Their cells are much distorted, show giant forms, multinucleation or syncytium formation and frequent mitoses are present. The Verocay bodies and palisaded nuclei seen in the better differentiated non-malignant analogue are wanting, or only hinted at in these. Collagenous tissue is not very abundant, the tone of the section in trichrome green stains being reddish, rather than green, as it would be in fibrous tumors. Occasionally one encounters strange composite tumors that may fall into this category, in which there are gland-like epithelial structures embedded in the tumor; these have been found in the groin and ascribed to rests from the embryonal nervous system.

Neurogenic sarcoma

There are two recognizable types of these, one of relatively benign appearance that usually arises near joints, or along the course of nerves and grows comparatively slowly; the other occurs at any point along a nerve trunk and more closely resembles an ordinary fibrosarcoma in every respect (fig. 6). The former class is easily mistaken for a rather cellular and atypical fibroma, but it is more metaplastic and its cells tend to grow in bundles, or leashes that show a peculiar whorled arrangement. These tumors tend to recur and, with each recurrence, to become more malignant in type. The second class differs little from any fibrosarcoma, but its tendency to form whorled bundles of parallel cells marks it off fairly well.

Malignant tumors in von Recklinghausen's disease

Here the malignant change is apt to occur in one of the larger tumors of the large crop of neoplasms. A neurofibroma may undergo dedifferentiation and become a neurogenic sarcoma, sometimes resembling a retothelial sarcoma strongly and suggesting the possibility of the development of a malignant type of the Antoni subdivision of the neurinoma. Myxosarcoma may also develop. It is a noteworthy fact that one frequently sees patients with this disease die of a malignant glioblastoma multiforme of the brain, which often develops in conjunction with von Recklinghausen's disease.

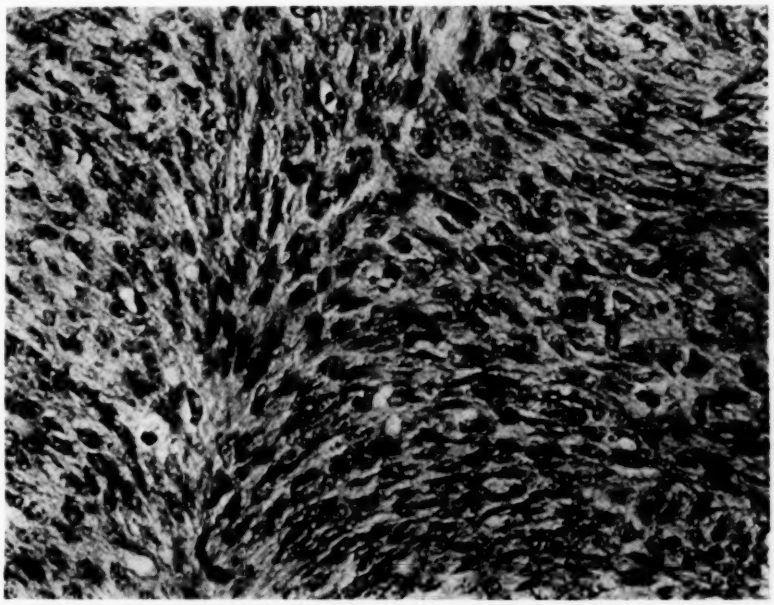

FIG. 6. MALIGNANT TUMOR, PROBABLY OF SCHWANN CELL ORIGIN. Known as "neurogenic sarcoma." It closely resembles a fibrosarcoma in its morphology, but in Masson sections stained with Lichtgrün, the predominating color is reddish, from the fuchsin and ponceau stain the Schwann cells and the sheath of Schwann, rather than greenish, as would be the case in a true fibrous tumor. (Masson trichrome lichtgrün.)

Malignant melanoma

The malignant analogue of the pigmented mole or nevus is often called simply "melanoma," which is misleading. It was formally termed "melanosarcoma" or "melanocarcinoma" according to the type of growth it showed (fig. 7). It arises from Schwann cells in Meissner corpuscles or in similar organs in the

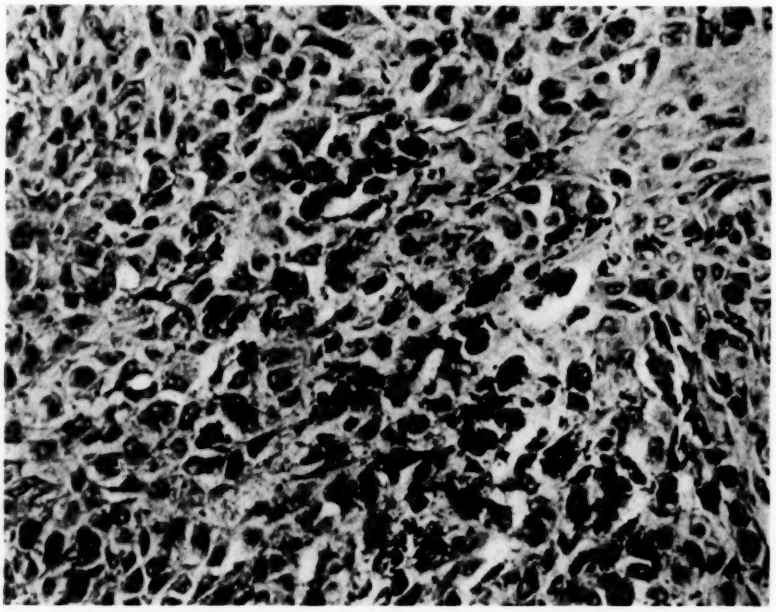

FIG. 7. SECTION FROM A LARGE MALIGNANT MELANOMA, OR "MELANOSARCOMA" OF THE RECTUM. This illustrates complete dedifferentiation of the type cells. The black masses and dots are the melanin contained in the tumor cells.

case of primary malignant melanoma of the meninges, (Foot and Zeek,[4]) and metastasizes widely and rapidly. It has perhaps the most sinister reputation among malignant tumors. One may suspect its presence in any very deeply pigmented mole that is rapidly growing larger. The transformation of the non-malignant mole into the malignant melanoma may sometimes be beautifully demonstrated in sections of such tumors removed at an

early stage of the process; I have seen two cases in which the superficial portions of the growth were quite innocent in appearance, but showed malignant transformation of the more deeply situated cell nests and invasion of the subcutaneous fat and lymphatics by these. Sometimes the malignant change occurs in isolated cell nests in the superficial portions of such tumors. It is only because of Masson's brilliant work on the histogenesis of the non-malignant pigmented nevus that we can deduce the same origin for the malignant cells; so metaplastic do they become and so rapidly do they multiply, that there is no time for differentiation and any connection between them and the terminal neurofibrillae cannot be made out, the tumor appearing carcinomatous or sarcomatous according to the type of growth it assumes. These neoplasms may show very great variations in their degree of pigmentation, some being quite black, others totally lacking in melanin ("amelanotic melanoma"); in some cases some of the metastases may be pigmented and others quite amelanotic. It is significant that these tumors always start where Meissner corpuscles occur. The histology of the malignant melanoma of the choroid of the eye is slightly different from that of those arising in the skin, but they seem to be closely related in either case. Malignant melanoma of the nail beds, or subungual melanoma, is sometimes encountered and is a very malignant form.

Malignant sympathetic nervous tumors

In mentioning ganglioneuroma I pointed out the necessity for bearing in mind the histogenesis of sympathetic ganglia. The tumors arising from elements of these ganglia have been subdivided by Scott[17] into three subtypes, depending upon the prevailing stage of development of the component cells. There are the large and general class of "sympathicoblastomas" (fig. 8) covering all of these; they were formerly called "neuroblastomas" by Wright,[18] but Bailey[1] renamed them to accommodate his class of true neuroblastomas of the central nervous system. These tumors usually arise in the suprarenal medulla, which also gives us a paraganglioma and a malignant "tumor of the adrenal

medulla" or maglinant paraganglioma. Clinically, if they metastasize to the liver they are known as the "Pepper type," while if they spread to the bones and the lungs they are called the "Hutchinson type" of sympathicoblastoma. The distinction appears to be largely a matter of mechanics in lymphatic or hematic spread. They are almost always seen in children and a mass near one

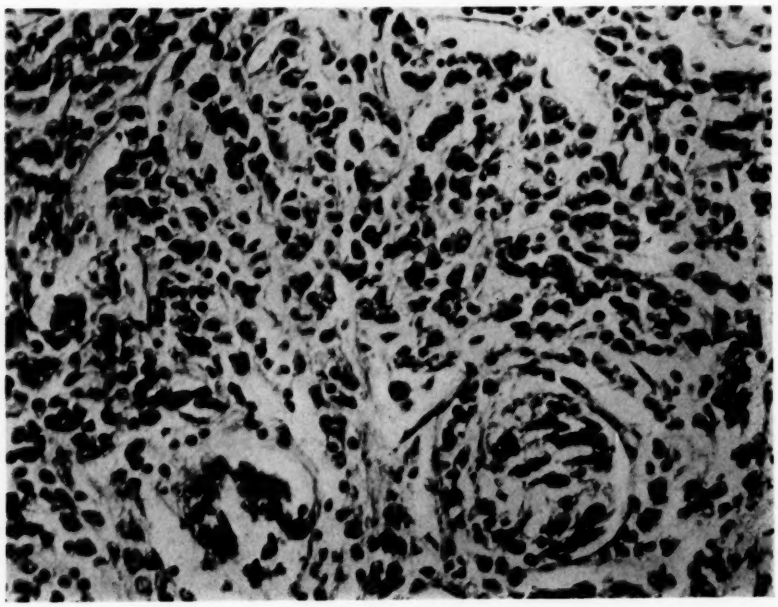

FIG. 8. MALIGNANT TUMOR OF SYMPATHETIC NERVOUS ORIGIN. The sympathicoblastoma or "neuroblastoma" of older literature. The cells have a tendency to form rosettes, not very well shown in this particular example, although pseudorosettes are present. This tumor was metastatic to a lymph node and originated in the suprarenal of a child. There is a superficial similarity to lymphosarcoma, for which these tumors were formerly mistaken. (Hematoxylin-eosin.)

kidney with metastases in lymph nodes and a skull riddled with soft, cushion-like growths should always arouse suspicion of this tumor. Histologically, the least differentiated tumors show an alveolar arrangement of cells resembling lymphocytes, but showing more cytoplasm, tails or processes in suitable preparations and a tendency to group themselves in rings, or pseudorosettes.

More differentiated forms show rosettes with an inner limiting membrane, like ducts, and cells resembling immature ganglion cells. Next come tumors with rosettes of cells differentiating toward capsule cells, surrounding bundles of glia fibers. Or these may be a differentiation toward the phaeochromocytes, or pigmented cells of the ganglia, in which case one speaks of a "phaeochromoblastoma" a tumor closely related to the paragangliomas. It would be well to point out that the subject at hand has merely been summarized and that there is a great deal of work to be done before the last word has been said. In such a paper as this classification stands out too boldly in relief in proportion to the really constructive material; it is usually much easier to classify than to understand tumors, or to explain their true nature and the subject matter is fitted together with a certainty that is not found in the interrelationship of the tumors with which it treats. If one classifies tumors in such a way as to make a sort of periodic table, like Mendelieff's, one may hope to find tumors ultimately that will fit into positions in this table as yet unoccupied. Such a table should be based upon embryological and histological data. One must remember, however, that the histology of the nervous system was not worked out by means of routine laboratory stains; it was the brilliant researches of men like Ramon y Cajal, Golgi, Nissl, Weigert and their like, employing a variety of fixing fluids and staining methods or impregnations with metals that have resulted in the present knowledge of the subject. Why hope to solve the equally complex riddles of neoplasia of these tissues by the aid of formalin fixed material and our old friends hematoxylin and eosin? That it can not be done is manifested by the progress that has been made since more selective methods were introduced into the pathologic laboratory.

It is to be hoped that nerve tumors will be produced in the course of the work with tumor "viruses," carcinogenic tars, hormones and the like, so that we may study them at and from their inception in experimental animals.

APPENDIX: TECHNIC

Great stress has been laid, in this paper, upon the importance of using a selective stain in connection with the more accurate diagnosis of neurogenic

tumors; it would, therefore, be well to give a brief resumé of the most useful stain for this purpose: the light green variant of the Masson trichrome method, a stain that dyes the nuclei violet to black, the cytoplasm rose red, the Schwann cells and erythrocytes coral red, muscle pink to bright red according to the fixative and all connective-tissue grass green. By this means it is easy to differentiate between fibrous and other tumors.

Fixation: Tissue may be fixed in any of the standard fixatives; good results may be obtained even in rapid routine fixation in 10 per cent formalin in 95 per cent alcohol; the chromium fixatives are probably the best, Zenker's fluid, or Masson's bichromate fixative which is 3 per cent bichromate of potash in water, to which 10 per cent strong formalin is added just before using. Sections from paraffin blocks are cut rather thin (as the stain is intense) and are then stained as follows:

Nuclear stain: (Regaud's iron hematoxylin.) One gram of hematoxylin is dissolved in 80 cc. of hot distilled water, the solution is cooled and 10 cc. of 95 per cent alcohol and 10 cc. of glycerol added. Sections are mordanted for 5 minutes in 5 per cent aqueous iron alum at 50°C. washed at the tap and stained for 5 minutes in hematoxylin solution at 50°C. As they are now black and overstained, they are differentiated in a saturated solution of picric acid in 95 per cent alcohol, 2 parts, to one part 95 per cent alcohol, until the nuclei alone retain the stain. They are then washed for 15 minutes in running water. One need not be too particular about getting all the hematoxylin out of the sections as a little in the connective tissue will not make them unpleasingly "muddy." Too much, however, will prevent the green stain from being clear and pure green.

Cytoplasmic stain: Sections stained as above are brought into a mixture of ponceau de xylidine (Krall) and acid fuchsin. Two parts of a 1 per cent aqueous solution of acetic acid with 1 per cent of the ponceau are mixed with one part of a similar acetic acid solution containing 1 per cent acid fuchsin. The acetic acid prevents the extraction of the dye in subsequent washings. The sections are stained in this for 5 minutes and then rinsed in distilled water.

Connective tissue stain: They are then mordanted for 5 minutes in 1 per cent aqueous phosphomolybdic acid, rinsed in distilled water and stained for 5 minutes in 2 per cent lichtgrün (Krall, or Grübler) in 2 per cent acetic acid, after which they are rinsed and placed in 2 per cent acetic acid for 2 minutes. They are then washed at the tap, brought up through ascending percentages of alcohol to xylol, in the usual manner and mounted in Canada balsam.

The dyes used are obtainable through Eimer and Amend in New York if they can not be readily procured locally. This method is very useful for orientation and will usually suffice for the identification of the various tumors mentioned in this paper. Silver impregnations, such as the Roger's method, are useful in demonstrating nerve fibers, axis cylinders, et cetera, but are not essential.

REFERENCES

(1) Bailey, P., and Cushing, H. W.: A classification of the tumors of the glioma group on a histogenetic basis with a correlated study of prognosis. Philadelphia: Lippincott, 1926, pp. 175.

(2) Ewing, J.: Neoplastic diseases. 3rd Ed. Philadelphia: W. B. Saunders, 1928, pp. 1127.

(3) Foot, N. C.: The Masson trichrome staining methods in routine laboratory use. Stain Techn., **8:** 101–110. 1933.

(4) Foot, N. C., and Zeek, P.: Two cases of melanoma of the meninges with autopsy. Am. Jour. Path., **7:** 605–618. 1931.

(5) Laidlaw, G. F.: Silver staining of the endoneurial fibers of the cerebrospinal nerves. Am. Jour. Path., **6:** 435–444. 1930.

(6) Laidlaw, G. F., and Murray, M. R.: Addenda to a theory of pigmented moles. Am. Jour. Path., **10:** 319–320. 1934.

(7) Laidlaw, G. F., and Murray, M. R.: Melanoma studies. III. A theory of pigmented moles. Their relation to the evolution of hair follicles. Am. Jour. Path., **9:** 827–838. 1933.

(8) Masson, P.: Les naevi pigmentaires, tumeurs nerveuses. Ann. d'anat. path. et d'anat. norm. méd-chir., **3:** 417–453. 1926.

(9) Masson, P.: Étude sur les glomus. Arch. per le sc. méd., **50:** 47–70. 1927.

(10) Masson, P.: Carcinoids (argentaffin-cell tumors) and nerve hyperplasia of appendicular mucosa. Am. Jour. Path., **4:** 181–212. 1928.

(11) Masson, P.: Contribution to the study of the sympathetic nerves of the appendix; the musculonervous complex of the submucosa. Am. Jour. Path., **6:** 217–234. 1930.

(12) Masson, P.: The significance of the muscular "stroma" of argentaffin tumors (carcinoids). Am. Jour. Path., **6:** 499–514. 1930.

(13) Masson, P.: Experimental and spontaneous Schwannomas (peripheral gliomas). Am. Jour. Path., **8:** 367–416. 1932.

(14) Masson, P., and Gery, L.: Les tumeurs glomiques sous-cutanées. en dehors des doigts. Ann. d'anat. path. et d'anat. norm. méd.-chir., **4:** 153–165. 1927.

(15) Maximow, A. A., and Bloom, W.: A textbook of histology, 1st ed. Philadelphia: W. B. Saunders, 1930, pp. 833.

(16) Popoff, N. W.: The digital vascular system with reference to state of glomus in inflammation, arteriosclerotic gangrene, diabetic gangrene, thromboangiitis obliterans and supernumerary digits in man. Arch. Path., **18:** 295–330. 1934.

(17) Scott, E., Oliver, M. G., and Oliver, M. H.: Sympathetic tumors of the adrenal medulla with report of four cases. Am. Jour. Cancer, **17:** 396–417. 1933.

(18) Wright, J. H.: Neurocytoma or neuroblastoma, a kind of tumor not generally recognized. Jour. Exp. Med., **12:** 556–561. 1910.

THE DETERMINATION OF BLOOD CHOLESTEROL*

I. A Comparison of Standard Colorimetric Methods and A Modified Method with Gravimetric Determination of Digitonin Precipitates

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For some time it has been recognized that various widely used colorimetric methods for determination of cholesterol in blood yield dissimilar results. As a consequence, the concentration of cholesterol in blood regarded as normal differs considerably depending on the method utilized for its determination. Regardless of the resulting confusion in interpretation of figures for blood cholesterol, no unanimity has been attained regarding the correctness of any procedure. However, gravimetric determination of cholesterol precipitated by digitonin under controlled conditions has been accepted by most workers as the method most nearly capable of yielding correct results. For this reason the accuracy of colorimetric procedures can be judged, to some extent, by the deviation from the results of gravimetric determinations carried out on the same samples. In the present study, results obtained by application of the widely used colorimetric methods of Myers and Wardell,[16] Bloor[3,4], and or Sackett's[19] adaptation of the Bloor technic have been compared with the results of the gravimetric technic of Windaus.[22] In addition, similar tests were made of a modified procedure developed by the authors in which sodium sulfate replaces the plaster of Paris employed in the method of Myers and Wardell. The standard colorimetric

*Modification of a paper read at a Symposium on Cholesterol before the Fourteenth Annual Convention of the American Society of Clinical Pathologists held at Atlantic City, June 7 to 9, 1935.

methods mentioned gave figures that differed to a significant degree with few exceptions from the simultaneously determined results of the digitonin method. The causes of these differences and means by which they may be eliminated or diminished are discussed in this and a subsequent paper.

EXPERIMENTAL

The method of Myers and Wardell[16] depends on extraction by chloroform of blood dried on plaster of Paris and application subsequently of the Liebermann-Burchard reaction. Determinations were done as directed by the authors except that in place of an artificial standard, standard solutions of pure cholesterol were employed, while 10 cc. of chloroform solution was treated in place of 5 cc. Subsequently, it was learned that substitution of the larger volume of chloroform might influence the results slightly, although any differences tended to diminish the error that has been observed. Plaster of Paris was obtained from two sources, either the general supply of the laboratory purchased in barrels, or, as in series 4 of table 1, from a sample of the best quality labeled "calcium sulfate, dehydrated, pure." Merck's Blue Label chloroform was used for the experiments described in this paper, while the acetic anhydride was analytical reagent quality employed without further purification.

According to the procedure outlined by Bloor[3,4] blood or other fluid is added to a mixture of alcohol and ether. Aliquot portions of the alcohol-ether extract are taken up in petroleum ether and again evaporated and dissolved in chloroform The Liebermann-Burchard reaction is then performed by addition of 2 cc. of acetic anhydride and 0.1 cc. of sulfuric acid to 5 cc. of the solution. The solutions are maintained at 20°C. for 15 minutes when colorimetric readings are made. Preliminary experiments on development of color demonstrated that more reproducible results could be obtained if readings were made 20 to 25 minutes after starting the reaction rather than 15 minutes as recommended by Bloor. This modification probably causes the method to give somewhat lower results because of the altered response of cholesterol bound as esters in contrast to uncombined cholesterol. Consequently, differences between the results of the Bloor method and of gravimetric determinations are also diminished. Temperatures were held at 20° during the development of color, while the solutions were exposed to the light of the lamp used for illuminating the colorimeter.

The Sackett[19] technic was similarly modified. The latter varies from the Bloor method in that the alcohol-ether solutions are not warmed while the extraction with petroleum ether also is discarded.

Saponification has been omitted from the Bloor procedure as we have performed it. This is in accord with the latest recommendation published in 1928,[4] when Bloor modified slightly his second technic originally published in 1916.[3]

An outgrowth of our investigation of the Myers and Wardell method was a simple modification of the procedure in which anhydrous sodium sulfate replaces plaster of Paris. 1 cc. of serum mixed in a mortar with about 8 grams of sodium sulfate was dried in an oven at 100° for 10 minutes, then cooled in a desiccator. The dried material after pulverizing was extracted with anhydrous chloroform in a continuous extracter of the type used by Stoner.[21] The volume of chloroform solution having been made to 25 cc., 5 cc. of the solution was treated with 2 cc. of a freshly prepared, warm, 10:1 mixture of acetic anhydride and sulfuric acid. Color development was then allowed to proceed for 25 minutes at a temperature of 30°–35°. The solutions were compared colorimetrically with a standard solution similarly treated. Since the Liebermann-Burchard reaction is governed by the quality of chloroform and acetic anhydride as well as other factors to be discussed, the combination of time, temperature, and reagent concentrations described may not be generally applicable. Conditions selected for carrying out the color reaction should be controlled preferably by tests made in each laboratory.

The use of sodium sulfate was suggested by the report of Hambleton[12] who found this substance superior to plaster of Paris as a dehydrating medium for use in extraction of lipids. Mueller[14] had previously employed anhydrous sodium sulfate for extraction of cholesterol from blood.

Gravimetric determinations were done according to the directions of Windaus[22] except that alcohol used for washing the precipitate was saturated with cholesteryl digitonide. Care was taken to adjust the concentration of excess digitonin required for complete precipitation of cholesterol to approximately optimal levels. The weight of precipitate was converted into figures for cholesterol by means of the factor 0.24316. It has been contended that saponification renders cholesterol partially non-precipitable by digitonin.[6] Many experiments were done to test the possibility of loss from this and other causes. Recovery of added cholesterol was complete within the experimental error of the method despite the fact that cholesterol had been subjected to the action of alkali in these experiments.

RESULTS

The results of simultaneous determinations by various methods are presented in table 1. The material analyzed was either serum of normal individuals employed in the laboratory or mixed plasma of hospital patients. Practically all figures are averages of duplicate or triplicate determinations.

A lack of agreement between the several methods is demonstrated. Thus Bloor's method gave results that average about 10 per cent higher than those of the gravimetric method. This difference is somewhat less than that observed by other authors.

TABLE 1

DETERMINATION OF CHOLESTEROL BY COLORIMETRIC METHODS COMPARED WITH GRAVIMETRIC DIGITONIN DETERMINATIONS

SAMPLE	DIGITONIN	SODIUM SULFATE	MYERS-WARDELL	BLOOR	SACKETT	SPECIMEN
			Series I			
A	155	160	125		165	Mixed
B	163	168	132		172	Mixed
Average.....	159	164	128.5		168.5	
			Series II			
3	160	155		178	175	Normal
4	143	132		171	169	Normal
5	245	238		273	269	Normal
16	173	184		183	172	Mixed
Average.....	180.3	177.3		201	196.3	
			Series III			
6	257	258		288		Normal
7	156	148		174		Normal
8	255	250		270		Normal
9	161	153		164		Normal
2	202	209		218		Mixed
Average.....	206.2	203.6		222.8		
			Series IV			
12	211	215	180			Normal
13	156	163	104			Normal
14	232	237	195	268		Normal
Average.....	199.7	205.0	159.7			
			Series V			
10	65		55			Post-natal
11	153		138			Mixed
15	156		118			Mixed
Average.....	124.7		103.7			

Mueller,[14] Feigl,[8] Stern and Suchantka,[20] and Mühlbock and Kaufmann,[15] have found the results obtained using Bloor's method to be high. Recently, Gibbs, Buckner, and Bloor[11] also have found differences between results of Bloor's colorimetric method and Okey's[17] oxidative digitonin method that averaged 30 per cent higher for the colorimetric determinations. In explanation, Bloor[4] infered that the digitonin precipitation method may be at fault. As explained previously, the modified procedure for developing color employed by us tends to give results somewhat lower than those obtained by the use of the original technic. Thus differences between the two methods would be diminished.

Sackett's modification of Bloor's procedure likewise yielded results that exceeded the gravimetric figures, although slightly lower than those of the original method. According to Bruger,[7] this statement applies also to values obtained by means of another modification, that of Mirsky and Bruger.[13]

Low results in comparison with parallel determinations by digitonin method were characteristic of the method of Myers and Wardell. Our findings differ in this respect from those of the authors of the method who found satisfactory agreement. Two explanations may be offered to account for this discrepancy. At the time that Myers and Wardell performed their experiments the need for adjusting the concentration of excess digitonin had not been recognized clearly and this factor may have influenced the results. Another factor of considerable importance is the quality of the plaster of Paris employed. Contrary to the conclusions of Andes[1] differences between the Myers-Wardell and gravimetric methods were greatest when a finely powdered, newly received lot of pure material was used. Another sample, plaster of Paris, technical, less pulverent and probably somewhat more hydrated since it had been in the laboratory for some time, gave somewhat smaller though still significant differences between the two methods. That one cause of the low results given by the Myers-Wardell procedure was incomplete extraction of cholesterol was convincingly demonstrated by experiments in which the residue of blood and plaster of Paris remaining after chloroform extraction was digested with 25 per cent potassium hydrox-

ide and subsequently extracted with ether in a continuous extracter. On removing the ether the material obtained dissolved in chloroform yielded appreciable amounts of color with the Liebermann reagents. It was evident therefore, that a considerable proportion of the cholesterol present had remained unextracted. Prolonging the time of extraction with chloroform as much as 3 or 4 hours had no appreciable influence on the results. Varying the time of drying similarly failed to bring about improvement.

Because of the poor results obtained when plaster of Paris was used, anhydrous sodium sulfate was substituted as outlined above. Reference to table 1 will show that introduction of this modification caused the results to agree reasonably well with those of the gravimetric technic. Only small amounts of cholesterol were recovered from the residue of serum and sodium sulfate remaining after extraction with chloroform when such residues were digested with potassium hydroxide and extracted as described. Thus loss by incomplete extraction was negligible.* The accuracy of the modified procedure is adequate for most clinical purposes. That it represents an improvement over the original is at once evident on comparing the deviations from the gravimetric figures.

In connection with our studies of the effect of sodium sulfate and plaster of Paris on extractability of serum cholesterol, other procedures for extraction of cholesterol were also examined. For complete extraction of cholesterol and other lipids from serum, the use of alcohol and ether as described by Bloor proved to be effective. The slight modification of Sackett does impair the efficiency somewhat. Extraction of serum dried on filter paper by chloroform or petroleum ether has always given low results in our hands. Forbes,[9] also Forbes and Irving,[10] have recom-

* While material giving the Liebermann-Burchard reaction, presumably cholesterol, could be detected in the residue after extraction, amounts were not sufficient to impair the utility of the method. It was assumed for a time that any loss was compensated by a positive error in colorimetry. However, the origin of such a compensating error, if it exists, has not been established. The writers are indebted to Mr. Fred Wilson for carrying out experiments establishing the presence of unextracted cholesterol in the sodium sulfate residue.

mended doucil, a synthetic zeolite, as a dehydrating medium for use in the extraction of cholesterol from blood. While the first sample of doucil tested gave results that were quite encouraging, subsequent samples failed to permit complete recovery of cholesterol. The procedure outlined by these authors is perhaps the simplest yet devised, however, further work is needed on standardization of the material before it can be applied with safety.

Saponification lowers the values obtained by colorimetric determination of cholesterol extracted by alcohol-ether and generally brings about better agreement with the results of the digitonin method. Among recent writers on the subject, Artom[2] has favored saponification prior to application of the Liebermann-Burchard reaction. However, Boyd[6] has pointed out that care must be exercised to extract completely cholesterol from alkaline residues after saponification. Neutralizing the alkali aids in accomplishing this. The effect of saponification in part is due to elimination of differences between free and esterified cholesterol in rate of development of color of the Liebermann-Burchard reaction. Other factors are involved, however, including the possibility that beside cholesterol, labile substances exist in serum that add to color of the Liebermann-Burchard reaction. While it has been suggested that some cholesterol may be rendered nonreactive in the course of saponification, we have not found evidence of such a conversion judged by precipitability of cholesterol by digitonin. Nevertheless, saponification must be conducted cautiously and the extra care and manipulation involved limit somewhat the adaptability of such procedures to clinical cholesterol determinations.

It is evident that none of the widely used standard colorimetric methods that have been tested in this study are wholly satisfactory for determination of cholesterol, while certain methods may give grossly inaccurate results. In general, when cholesterol is completely removed from serum by means of hot alcohol-ether the results of colorimetric determinations are high compared with those of the gravimetric method. On the other hand, low values characterize the procedures that involve drying of blood

preliminary to extraction apparently because some of the cholesterol resists extraction under these circumstances. Contrary to the rule, cholesterol is efficiently extracted by anhydrous chloroform from serum dried on anhydrous sodium sulfate. When serum cholesterol so obtained is quantitated by means of the properly regulated Liebermann-Burchard reaction, values are obtained that agree reasonably well with those found by gravimetric digitonin determinations.

SUMMARY

Several widely used colorimetric methods for determination of cholesterol have been tested by comparing the results with those of simultaneous determinations by the gravimetric digitonin method. Significant deviations from the latter were observed. Differences were greatest when serum was dried on plaster of Paris and extracted with chloroform prior to development of color by the Liebermann-Burchard reaction. Incomplete extraction of cholesterol explains the low values so obtained. Anhydrous sodium sulfate substituted for plaster of Paris permits complete extraction of cholesterol from dried serum.

High values characterized methods depending on extraction by alcohol and ether when development of color was carried out by the usual technic. Results yielded by these methods were lowered by saponification of the alcohol-ether extract.

REFERENCES

(1) Andes, J. E.: A modified method for the determination of blood cholesterol. Jour. Lab. and Clin. Med., **20:** 410–415. 1935.
(2) Artom, C.: Fat metabolism. Ann. Rev., Biochem., **4:** 199–224. 1935.
(3) Bloor, W. R.: The determination of cholesterol in blood. Jour. Biol. Chem., **24:** 227–231. 1916.
(4) Bloor, W. R.: The determination of small amounts of lipid in blood plasma. Jour. Biol. Chem., **77:** 53–73. 1928.
(5) Bloor, W. R.: Fat metabolism. Ann. Rev., Biochem., **3:** 175–192. 1934.
(6) Boyd, E. M.: Diurnal variations in plasma lipids. Jour. Biol. Chem., **110:** 61–70. 1935.
(7) Bruger, M.: Personal communication.
(8) Feigl, J.: Über Cholesterinämie. I. Vergleichende Untersuchungen zur Methodik und besonderer Berücksichtigung der colorimetrischen Verfahren. Ztschr. f. d. ges. exper. Med., **11:** 178–238. 1920.

(9) FORBES, J. C.: Determination of cholesterol in blood plasma and serum. Jour. Lab. and Clin. Med., **16:** 520–521. 1931.

(10) FORBES, J. C., AND IRVING, H.: The determination of cholesterol in whole blood. Jour. Lab. and Clin. Med., **16:** 909–912. 1931.

(11) GIBBS, C. B. F., BUCKNER, E., AND BLOOR, W. R.: The cholesterol to cholesterol ester ration in the plasma of diabetics with advanced arteriosclerosis. New Eng. Jour. Med., **209:** 384–384. 1933.

(12) HAMBLETON, A.: A Soxhlet type of extraction apparatus for operation at low temperature under reduced pressure. Jour. Biol. Chem., **99:** 289–294. 1932.

(13) MIRSKY, I. A., AND BRUGER, M.: A note on the Liebermann-Burchard color reaction for blood cholesterol. Jour. Lab. and Clin. Med., **18:** 304–306. 1932.

(14) MUELLER, J. H.: A comparison of the results obtained by the colorimetric and gravimetric determinations of cholesterol. Jour. Biol. Chem., **25:** 549–560. 1916.

(15) MÜHLBOCK, O., AND KAUFMANN, C.: Die gravimetrische Cholesterinbestimmung im Blut und Serum. Biochem. Ztschr., **233:** 222–235. 1931.

(16) MYERS, V. C., AND WARDELL, E. L.: The colorimetric estimation of cholesterol in blood, with a note on the estimation of coprosterol in feces. Jour. Biol. Chem., **36:** 147–156. 1918.

(17) OKEY, R.: A micromethod for the estimation of cholesterol by oxidation of the digitonide. Jour. Biol. Chem., **88:** 367–379. 1930.

(18) REINHOLD, J. G.: Quantitative determination of free cholesterol and of cholesterol as esters without digitonin. Proc. Soc. Exper. Biol. and Med., **32:** 614–616. 1935.

(19) SACKETT, G. E.: Modification of Bloor's method for the determination of cholesterol in whole blood or blood serum. Jour. Biol. Chem., **64:** 203–205. 1925.

(20) STERN, R., AND SUCHANTKA, G.: Über die klinische Bedeutung des Cholesterins in der Galle und im Blutserum. Arch. exper. Path. u. Pharm., **115:** 221–231. 1926.

(21) STONER, W. H.: Quoted from HAWK, P. B., AND BERGEIM, O. Practical physiological chemistry, 10th ed. Philadelphia: P. Blakiston and Sons, p. 441. 1931.

(22) WINDAUS, A.: Über die quantitative Bestimmung des Cholesterins und der Cholesterinester in einigen normalen und pathologischen Nieren. Ztschr. f. physiol. Chem., **65:** 110–117. 1910.

THE DETERMINATION OF BLOOD CHOLESTEROL*

II. Factors Influencing the Accuracy of Various Methods

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The correct determination of cholesterol has proved to be a troublesome problem particularly for the clinical laboratory. For reasons of speed, sensitivity and simplicity the use of colorimetric procedures has been practically obligatory. However, colorimetric methods fail to yield concordant results and are frequently susceptible to random errors. A critical investigation of certain cholesterol methods has been made with the object of discovering the cause of these errors. My experiments have enabled me to evaluate various factors important for accurate cholesterol determinations. In addition, a study of the Liebermann-Burchard reaction has provided a basis for a new method for estimating cholesterol esters in which the use of digitonin is eliminated. The present paper summarizes the results of these studies with special reference to their bearing on the determination of cholesterol in the clinical laboratory. While the primary object is to discuss colorimetric technic, an adequate examination of the subject involves also some consideration of methods that are based on precipitation by digitonin. No attempt has been made to review comprehensively the literature dealing with the determination of cholesterol as the subject has been surveyed recently by Wasitzky.[25]

All methods obviously are dependent on the efficiency of the process employed for the extraction of cholesterol from blood. Faulty procedures used for this purpose explain the low results

* Modification of a paper read at a Symposium on Cholesterol before the Fourteenth Annual Convention of the American Society of Clinical Pathologists held at Atlantic City, June 7 to 9, 1935.

of certain methods as shown by Reinhold and Shields.* The addition of blood or other fluid to alcohol and ether with subsequent warming is efficient but leads to high results with the usual colorimetric technic. Extraction of cholesterol by chloroform from plasma dried on anhydrous sodium sulfate is only slightly less effective. It offers also the advantage of convenience and perhaps of greater specificity, besides accomplishing removal of certain interfering substances.

For some purposes, determination of cholesterol by precipitation with digitonin is desirable. The advantages are higher precision and ability to separate free and esterified cholesterol. Presumably also the method is more specific for cholesterol, but this remains somewhat uncertain. While macro-gravimetric methods undoubtedly are more accurate than other methods, these methods are seldom used clinically for blood, because of the excessive requirements for material. Micro-gravimetric methods are better adapted for this purpose, and cholesterol in as little as 2 cc. of serum can be determined accurately by any of several technics. I have found that of Ewert[4] to be satisfactory. A micro-balance is not essential. Results obtained by gravimetric digitonin methods must be corrected according to the excess of digitonin present at the time of precipitation. Ewert has included in his paper a chart for correcting results to those given by an optimal excess of digitonin, although according to Schoenheimer and Dam,[23] the composition of cholesterol digitonid may differ with various preparations of digitonin.

Cholesterol precipitated by digitonin may be determined also by oxidation and titration or by colorimetry as alternatives to gravimetry. Oxidation procedures require meticulous technic and considerable practice before they may be applied successfully. Schoenheimer and Sperry[24] have applied the Liebermann reaction to the determination of cholesterol precipitated as the digitonid. While oxidation methods are subject to the influence of variations in the composition of cholesterol digitonid, this source of error is avoided in the Schoenheimer-Sperry method provided sufficient

* This Journal.

digitonin is used to precipitate all of the cholesterol. As described by the authors, their method utilizes photometry; however, Fitz[7] and also Shapiro, Lerner and Posen[21] have adapted it to the colorimeter. Nevertheless, a higher degree of technical skill and greater expenditure of time are required as compared with direct colorimetry without preliminary precipitation of cholesterol.

Practicaly all colorimetric methods for cholesterol have employed the acetic anhydride-sulfuric acid reaction of Liebermann as applied by Burchard. Although it is quite possible that other color reactions may prove superior, this reaction is capable of yielding accurate analytical data. The Liebermann-Burchard reaction can be divided conveniently into several stages. The color at first is predominantly blue-green (disregarding transitory red, purple and blue hues often seen immediately after initiating the reaction). The duration of this stage depends largely on the temperature. Gradually the blue element gives way to yellow and the subsequent stage may be characterized as yellow-green. Eventually, after a number of hours, the green hue disappears and the solutions become yellow. These changes have been followed spectrophotometrically by Morgareidge.[16]

The results obtained when the Liebermann-Burchard reaction is applied to solutions of blood lipid are accepted as a measure of the blood cholesterol, although it is a well recognized fact that the reaction is not specific for cholesterol. Numerous compounds form colors when treated with the reagents (La Lande,[9] Levine and Bien,[10] Levine and Richmond,[11] Ruzicka et al.[19]). The use of a red filter in the colorimeter eliminates interference from substances giving a red color should these occur in blood serum. For other reasons also we have found a red filter to be advantageous in connection with cholesterol determinations.

A number of authors (Luden,[14] Mueller,[17], Feigl,[5] Lifschütz[12,13]) have claimed that other substances in blood contribute to the color developed when blood cholesterol reacts with sulfuric acid and acetic anhydride in the presence of chloroform. Evidence presented to support these statements has been of an indirect nature. Bloor and Knudson[3] found no evidence to uphold these claims, and while not denying the possible existence of such sub-

stances, stated that if they existed they behaved with digitonin as did ordinary cholesterol. Gardner and Gainsborough[8] likewise could not establish the presence of additional sterol or related compounds in non-saponifiable residues obtained in the course of blood cholesterol determinations. My interest in this question was stimulated particularly by the fact that a green precipitate separated from chloroform solutions of serum cholesterol under the conditions used for the determination of cholesterol by the method previously described.[18] The nature of the material so obtained has not been established; however, similar precipitates have never been detected in solutions of pure cholesterol or of cholesterol esters under like circumstances. Possibly the green precipitate consists merely of a colored derivative of cholesterol entrained on a colorless precipitate. On the other hand it may actually indicate the existence of an additional reactive substance that contributes to the Liebermann-Burchard reaction of blood serum sterol.

Ordinarily the precipitate does not appear or is reduced in amount when plasma is dried on sodium sulfate and extracted with chloroform. After saponification the bulk of the precipitate is diminished, while the color is changed. A similar precipitate fails to appear when more than minimal concentrations of sulfuric acid are employed, although solutions so treated frequently become turbid on standing.

While the consistently high results of colorimetric methods that employ the alcohol-ether extraction procedures might be explained by the presence of additional reacting substances, it is questionable whether more than a fraction of the apparent excess of color may be attributed to lack of specificity of the reaction. Differences in the Liebermann-Burchard reaction of free cholesterol and of cholesterol bound as esters, are a potent source of error that hitherto has been unrecognized. Cholesterol combined as esters reacts with a greater velocity than free cholesterol and under certain conditions may yield color of greater intensity. Thus colorimetric comparisons of solutions containing both free and esterified cholesterol with standard solutions containing only free cholesterol would result in high readings for the former. Low

temperatures and small concentrations of sulfuric acid accentuate the dissimilarity, while increased concentrations of sulfuric acid and high temperatures have the opposite effect. The difference may persist during the early phases of the Liebermann-Burchard reaction, but diminishes as the reaction advances. During the later stages true color equivalence exists between free and esterified cholesterol. Whether color equivalence will be attained within the time permitted for development of color in standard cholesterol methods, will depend on the elevation of temperature brought about by mixing the reagents, on the rate of cooling and on the quality of chloroform and acetic anhydride. Small details of technic thus may influence the results appreciably. However, the effect of the difference in reactivity of free and of esterified cholesterol, is easily overcome by suitable regulation of reaction time and temperature. Thus heat generated on addition of acetic acid and sulfuric acid may raise the temperature enough to bring about color equivalence within five to fifteen minutes provided the tubes are not cooled during this time. A more dependable means of accelerating the reaction is warming the solution in a water bath.

Solutions of cholesterol treated according to the several directions of Bloor[1,2] have been found to give 10 to 20 per cent less color when read after fifteen minutes than did equivalent concentrations of cholesteryl oleate or palmitate. Frequently, depending on the chloroform used, color equivalence was not realized at any time during the reaction, although ordinarily colorimetric readings were practically identical after twenty-five to thirty minutes. Undoubtedly the differences observed at fifteen minutes when readings are made in Bloor's technic explain in part the high results that characterize this method. The temperature control procedure of Mirsky and Bruger[15] permits a greater temperature rise than does the Bloor technic, so that the reactions of free and esterified cholesterol are somewhat more uniform. However, the results of this modified technic also are susceptible to error from failure to obtain color equivalence.

The changed response of cholesterol following esterification has been verified experimentally. The degree of divergence from the

course of the reaction of cholesterol depends on the chloroform employed. Differences appear to be greatest in anhydrous chloroform of low alcohol content. Evaluation of the maximal color developed by each solution tested as suggested by Fidler[6] will aid somewhat in securing correct colorimetric values. However, it fails to overcome error caused by inequalities in the depth of color.

The difference in the velocity and intensity of the early stages of the Liebermann-Burchard reaction of esterified cholesterol as compared with free cholesterol can be utilized for the determination of these fractions of serum cholesterol.[18] The greatest differences are observed when the reaction is carried out in the cold with minimal concentrations of sulfuric acid. Under these conditions, solutions of cholesterol esters develop enough color to permit colorimetric measurements. Free cholesterol on the other hand, forms but little color, and correction may be made for the slight color so contributed. Known solutions of cholesteryl oleate or cholesteryl palmitate are used as standards. Not all cholesterol esters appear to behave alike under these conditions, therefore the naturally occurring esters of blood are employed.

After reading the color formed by esterified cholesterol in the cold, the same solutions may be utilized for total cholesterol determinations by warming to 35°–38° for 40 to 60 minutes. As already mentioned, both forms of cholesterol react under these conditions to give equivalent concentrations of color. Since determination of cholesterol esters by this procedure represents only an additional step in the determination of total cholesterol, the new method should permit more widespread application of cholesterol ester determinations.

Although a small amount of alcohol in chloroform is desirable, an excess slows the Liebermann-Burchard reaction and also diminishes the amount of color that is formed. The reaction of cholesterol combined as esters appears to be influenced to a greater extent than the reaction of free cholesterol, hence the concentration of alcohol and of water must be minimal if the two forms of cholesterol are to be differentiated by the colorimetric method described. On the other hand, cholesterol determinations as they

are done ordinarily depend to a considerable extent on the heat developed when alcohol and water with chloroform react with the reagents. The rise in temperature must be sufficient to accelerate the reaction of free cholesterol to the point where color developed is equivalent to that given by esterified cholesterol. Employing standard methods for total cholesterol, better results were obtained by means of chloroform U.S.P. than when chloroform free of alcohol and of water was used. The opposite is true when the two forms of cholesterol are to be differentiated.

Impurities in chloroform may cause large errors in all colorimetric methods. Ordinarily, commercial chloroform contains enough alcohol to prevent formation of decomposition products; however, samples are encountered occasionally that contain phosgene, peroxides, chlorine or hydrochloric acid. Highly purified chloroform is particularly susceptible to decomposition. The presence of phosgene and other oxidation products of chloroform, decreases the intensity of the color formed. Frequently the color is of a brown or yellow cast. Chloroform may be tested for the presence of its decomposition products by overlaying a small amount with baryta water. No film should form at the interface.

Complete absence of moisture from reagents and apparatus is essential for accurate colorimetric determination of cholesterol. The insidious influence of moisture on the Liebermann-Burchard reaction is so well known that comment is scarcely needed, yet I have found that despite the use of anhydrous reagents, moisture in injurious concentration may be introduced by condensation when volatile solvents are exposed to evaporation. Working in a region where atmospheric humidity is frequently elevated, it is necessary at such times to warm glassware and solvents when the latter are exposed. It should be mentioned that acetic anhydride must be of the best quality or freshly distilled. Chromogenic power varies widely and deteriorates on long standing.

It is the blue green stage of the Liebermann-Burchard reaction that is so markedly influenced by moisture. The yellow green stage is relatively immune to this effect according to our experience and that of Silink[22] as well. The great majority of workers

have employed the former for colorimetry of cholesterol although Schube[17] has recommended the use of the final yellow color.* However, utilization of the yellow-green color for this purpose, aside from overcoming the effects of moisture may be advantageous for other reasons as well. The influence of temperature, illumination and concentration of reagents is diminished. The color is quite stable, while that of the early stage is notoriously changeable. According to Silink, color intensity during the period of fading color, that is, the transition to yellow-green and yellow, is more constant and reproducible than during the preceding period of increasing color. Furthermore it is during the blue-green phase that cholesterol when combined as esters yields more color than an equivalent quantity of free cholesterol. As pointed out previously, this difference becomes negligible or disappears in the course of transition to the yellow-green stage. Finally, when the yellow-green stage is used for colorimetric determination of cholesterol, the results obtained approximate values found by the use of digitonin.

SUMMARY

Methods for the determination of serum cholesterol have been surveyed from the standpoint of errors involved in the various procedures. The lack of specificity of the Liebermann-Burchard reaction for cholesterol and the possible import of a green precipitate that appears under certain conditions when serum cholesterol reacts have been discussed. The behavior of cholesterol combined has esters when treated with the Liebermann-Burchard reagents differs from that of free cholesterol, the former reacting with a greater velocity to form color of greater intensity during the first (blue-green) stage of the reaction. Since many methods utilize this stage of the reaction for colorimetry, it is evident that this difference in response may constitute a significant source of error. This complication as well as others resulting from the presence of traces of moisture, impermanence of color, and the

* The results reported by Schube [20] range well below figures found in similar series by the use of the digitonin method. Incomplete extraction contingent to the use of plaster of Paris as a dehydrating agent accounts for this discrepancy.

effects of temperature, illumination, and reagent quality and concentration are avoided or minimized by use of the yellow-green color of the more advanced stage of the Liebermann-Burchard reaction for colorimetry. The quality of the chloroform employed is highly important for accurate determinations by means of the Liebermann-Burchard reaction.

REFERENCES

(1) Bloor, W. R.: The determination of cholesterol in blood. Jour. Biol. Chem., **24:** 227–231. 1916.

(2) Bloor, W. R.: The determination of small amounts of lipid in blood plasma. Jour. Biol. Chem., **77:** 53–73. 1928.

(3) Bloor, W. R., and Knudson, A.: The separate determination of cholesterol and cholesterol esters in small amounts of blood. Jour. Biol. Chem., **27:** 107–112. 1916.

(4) Ewert, B.: Über gravimetrische Cholesterin-bestimmung und eine Methode zur Cholesterinanalyse von Organgeweben. Biochem. Ztschr., **263:** 149–165. 1933.

(5) Feigl, J.: Cholesterinämie. I. Vergleichende Untersuchungen zur Methodik und besonderer Berücksichtigung der colorimetrischen verfahren. Ztschr. f. d. ges. exper. Med., **11:** 178–238. 1920.

(6) Fidler, R. S.: A simplified technique for the colorimetric determination of blood cholesterol. Jour. Lab. and Clin. Med., **20:** 307–311. 1934.

(7) Fitz, F.: The application to the colorimeter of the Schoenheimer and Sperry method for the determination of total and free cholesterol. Jour. Biol. Chem., **109:** 523–527. 1935.

(8) Gardner, J. A., and Gainsborough, H.: Studies in the cholesterol content of human plasma. V. The nature of the sterols of blood-plasma. Jour. Biochem., **28:** 1635–1639. 1934.

(9) LaLande, W. A., Jr.: Resin studies. II. A study and extension of the Liebermann color reaction for abietic acid. Jour. Am. Chem. Soc., **55:** 1536–1540. 1933.

(10) Levine, V. E., and Bien, G. E.: Liebermann-Burchard reaction with carotene. Proc. Soc. Exper. Biol. and Med., **31:** 804–808. 1934.

(11) Levine, V. E., and Richman, E.: Liebermann-Burchard reaction with compounds containing 5 membered monoheterocyclic rings. Proc. Soc. Exper. Biol. and Med., **31:** 582–584. 1934.

(12) Lifschütz, I.: Die Abbauprodukte des Cholesterins in den tierischen Organen. Ztschr. f. Physiol. Chem., **117:** 201–217. 1921.

(13) Lifschütz, I.: Zur Kenntnis der chemischen Natur und der Wandlungen des Blutfettes. Ztschr. f. Physiol. Chem., **117:** 212–217. 1921.

(14) LUDEN, G.: Studies on cholesterol. III. The influence of bile derivatives on Bloor's cholesterol determination. Jour. Biol. Chem., **29:** 463–476. 1917.

(15) MIRSKY, I. A., AND BRUGER, M.: A note on the Liebermann-Burchard color reaction for cholesterol in blood. Jour. Lab. and Clin. Med., **18:** 304–306. 1932.

(16) MORGAREIDGE, K.: Spectrophotometric studies on the Liebermann-Burchard reaction for sterols. Jour. Biol. Chem., **109:** lxvii–lxviii. 1935.

(17) MUELLER, J. H.: A comparison of the results obtained by the colorimetric and gravimetric determinations of cholesterol. Jour. Biol. Chem., **25:** 549–560. 1916.

(18) REINHOLD, J. G.: Quantitative determination of free cholesterol and cholesterol as esters without digitonin. Proc. Soc. Exper. Biol. and Med., **32:** 614–616. 1935.

(19) RUZICKA, L., GOLDBERG, M. W., AND WIRZ, H.: Sexualhormone. III. Zur Konstitutionsaufklärung des Androsterons. Helv. Chim. Acta, **18:** 61–68. 1935.

(20) SCHUBE, P. G.: A method of measuring cholesterol. Jour. Lab. and Clin. Med., **18:** 306–309. 1932.

(21) SHAPIRO, A., LERNER, H., AND POSEN, E.: A fixed color standard for cholesterol determinations. Proc. Soc. Exper. Biol. and Med., **32:** 1300–1301. 1935.

(22) SILINK, K.: Contributions to the biochemistry and physiology of cholesterol. I. Technique of micro analysis of cholesterol. Microchemie, **16:** 45–66. 1934.

(23) SCHOENHEIMER, R., AND DAM, H.: Über die Spaltbarkeit und Löslichkeit von Sterindigitoniden. Ztschr. Physiol. Chem., **215:** 59–63. 1933.

(24) SCHOENHEIMER, R., AND SPERRY, W. M.: A micro method for the determination of free and combined cholesterol. Jour. Biol. Chem., **106:** 745–760. 1934.

(25) WASITZKY, A.: Der Nachweis und die Bestimmung kleiner Mengen von cholesterin und anderen Sterinen. Microchemie, **14:** 289–310. 1934.

FURTHER STUDIES IN EXPERIMENTAL GRANULOPENIA*

With Particular Reference to Sulphydryl (Glutathione) Metabolism in Blood Dyscrasias

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We have presented evidence in previous publications[22,23,24] to indicate that the disease, agranulocytosis, is caused by certain drugs which have the benzene ring as their nucleus.

In our previous studies we have shown that the introduction of pure benzene subcutaneously or intraperitoneally in small daily doses, will result in the development of agranulocytosis in rabbits and we have long held that the granulopenic effect of drugs is due to the oxidation of this benzene nucleus, and that the effect on the bone marrow is due directly to one of these oxidation products.

In support of this conception we have shown that it is possible to oxidize amidopyrine in vitro, under conditions simulating those in the stomach, and in this way produce certain oxidation products, notably catechol and quinone. When these products are injected into rabbits, there follows the development of agranulocytosis in an occasional animal.

From these studies the question arises as to why leukopenia is produced in only an occasional animal, and it is the purpose of this paper to throw further light on that question, and also to attempt to show why only the occasional person develops this disease when many thousands of people are habitual users of drugs containing the benzene ring.

For example, we have been able to produce the disease fairly consistently with small doses of benzene, yet some of our experi-

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mental animals have tolerated daily doses for a period of months without showing ill effects.

This leads then to a consideration of the mechanism of defense in the rabbit against benzene poisoning, and in this connection we have studied the bone marrow of a large number of "benzene leukopenic rabbits," and we have found that the basic pathologic state is a hypoplasia or even an aplasia of the granulocytic elements of the red marrow. We consider bone marrow hypoplasia to be a quantitative decrease in the amount and number of granulocytic immature cell types, and probably this is brought about by a retardation of the rate of cell division. Furthermore, it is probable that this is also the same bone marrow picture that is seen in human agranulocytosis.[4,23] There are some who believe that the bone marrow shows a "hyperplasia," evidenced by a quantitative increase in the early blast forms with a decrease in the more mature marrow types, this being brought about, presumably by an arrest of maturation at the myeloblastic level.[8]

If the agranulocytic bone marrow is characterized by retardation of the rate of cell division, the important question is, what agent is responsible for this action.

At this time the one agent that seems actively concerned with cell division in the animal body is the sulphydryl group, which is found in the animal body, chiefly in the form of glutathione.

Glutathione is a chemical agent that was first observed in the tissues by deRey Pailhade[9] in 1888, and later given its present name by Hopkins.[18] In 1925 Holden[17] demonstrated its presence in blood cells and its absence in the blood plasma. Hunter and Eagles[20] first isolated the substance from human blood cells. After this, much investigation was directed toward the elucidation of its chemical structure and physiological properties, with final agreement that the active structural unit is the SH or reduced sulphydryl group. It seems agreed further that the action of this substance is dependent upon the oxidation reduction complex, (2GSH GS-SG) which is autocatalytic and reversible.[15,21]

Renewed interest in glutathione was stimulated by the work of Hammett and Hammett[11,12,13,14] who demonstrated the accelerating effect of reduced sulphydryl on the rate of cell division in

the regenerating chela of the hermit crab. They showed further that if the reduced sulphydryl was oxidized to the SS form, this acceleration activity was lost, and that further oxidation resulted in forms which were actually inhibitory to the rate of cell division. Reimann[30,31,32,33] in his attempts to apply these observations to the etiology of cancer, found that reparative cell growth in old and indolent ulcers could be stimulated by the application of the reduced form. All of these workers have emphasized that the

TABLE 1

GLUTATHIONE CONTENT OF BLOOD AND TISSUES OF NORMAL RABBITS, AS REPORTED BY OTHER AUTHORS

AUTHOR	BLOOD	LIVER	SPLEEN
	mgm./per cent	*mgm./per cent*	*mg./per cent*
Brown and Kolmer[6]	37	255	244
Matsumori and Okuda[27]		262	260
Binet and Arnaudet[3]		250	
Varela, B. et al.[36]	49	223	357
	50	305	350
	40	209	325
		255	369
Litarczek et al.[26]	26.2		
	23.5		
	26.7		
	33.6		
	19.3		
Kushiyama[25]	23	270	191 (Bone marrow–80)
Hongo[19]		216	

reduced form is the biologically active agent, regardless of its chemical attachments.

DISTRIBUTION OF SH IN ANIMAL TISSUES

In the animal body, the reduced-oxidized sulphydryl complex is found mainly in the form of glutathione, which has been demonstrated in varying amounts in all tissues. However, those tissues which are more cellular, contain the largest quantity. Many workers have endeavored to ascertain the normal content of the

various tissues with considerable variation in results, leading, therefore, to the acceptance of a range of normal for the different organs and tissues, which is given in table 1.

In a study of glutathione changes in the hematologic states, we are concerned mainly with its distribution in the bone marrow, spleen, liver, and blood. Due to a paucity of reports of glutathione content in the above named tissues, we found it necessary to establish normal figures in rabbits, and our findings are given in table 2. These findings are in agreement with the few reports in literature. In this work all determinations were made according to the iodometric method of Woodward and Fry.[39]

GLUTATHIONE DISTRIBUTION IN "BENZENE LEUKOPENIC RABBITS"

The chief purpose of the experiment given below was to determine if any alteration of glutathione content occurred in the liver, spleen, bone marrow and blood of rabbits made leukopenic with benzene, and these four tissues were selected because of their probable importance in the general problem of hematopoiesis.

From a study of table 2, it can be seen that the average glutathione in the rabbit bone marrow is 76 mgm./per cent, 323 mgm./per cent in the liver, 425 mgm./per cent in the spleen, and 34.5 mgm./per cent in the blood. These figures for the tissues indicate only the reduced form, but since the oxidized form also is found in the blood, the figures in the table give the amounts of both types.

Due to the fact that the blood glutathione is present only in the cells, true comparative values can be computed only by the calculation of a quotient which considers the number of erythrocytes present at the time of determination and for this purpose, Gabbe's quotient was calculated, which consists of dividing the amount of glutathione by the erythrocyte count.

Glutathione determinations, erythrocyte, and leukocyte counts were carried out on the heart's blood of eleven normal rabbits. The animals were given daily subcutaneous injections of 1 cc. of benzene in 0.2 cc. of olive oil, and the injections continued until the animals developed marked leukopenia, usually to below 1000 cells per cubic millimeter. They were then bled from the heart,

killed by air injection, and glutathione determinations carried out on the blood, liver, spleen, and bone marrow. From a study of these experiments certain changes were evident: the reduced glutathione of the blood was markedly decreased in ten of the eleven

TABLE 2

GLUTATHIONE IN BLOOD AND TISSUES OF NORMAL RABBITS*

RABBIT	BLOOD				BONE MARROW	LIVER	SPLEEN
	R.G.	R.Q.	T.G.	T.Q.			
1	32.0	6.0	64.0	12.0	61.0		
2	30.0	5.0	42.0	8.0	80.0	382.0	473.0
3	23.0	3.0	49.0	7.0	111.0	306.0	479.0
4	27.0	4.0	51.0	8.0	75.0	317.0	397.0
5	18.0	3.0	41.0	6.0	55.0	288.0	352.0

* Results other than quotients expressed in mgm./per cent.

R.G.—reduced glutathione, R.Q.—quotient of reduced glutathione, T.G.—total glutathione, T.Q.—quotient of total glutathione.

TABLE 3

COMPARISON OF AVERAGE GLUTATHIONE CONTENT IN BLOOD AND TISSUES OF NORMAL AND LEUKOPENIC RABBITS

BLOOD	NORMAL RABBITS	LEUKOPENIC RABBITS*
Reduced glutathione.....	34.5 mg. (24 animals)	13.0 mg. (11 animals)
Total glutathione.....	47.0 mg. (24 animals)	41.0 mg. (11 animals)
Reduced quotient.....	6.0 (24 animals)	2.7 (11 animals)
Total quotient.....	8.8 (24 animals)	9.5 (11 animals)
Bone marrow.....	76.0 mg. (5 animals)	56.3 mg. (11 animals)
Liver.....	323.0 mg. (4 animals)	380.0 mg. (11 animals)
Spleen.....	425.0 mg. (4 animals)	364.0 mg. (11 animals)

Results other than quotients expressed in mgm./per cent and represent the average amounts in the number of animals indicated.

* Average leukocyte count at time of determinations, with complete granulopenia in every instance, 740.

animals, and the quotient correspondingly reduced. The average reduced glutathione fell from 35 to 13 mgm./per cent and the quotient from 6.3 to 2.7. The total was little affected, indicating a conversion from the reduced to the oxidized state. Also, the bone marrow glutathione was reduced to 56 mgm./per cent, as com-

pared to a series of five normal animals, with an average of 76 mgm./per cent. The content of the other tissues showed no consistent alterations. Table 3 shows the findings in condensed form, with the normal figures for comparison.

It seems, therefore, that in the "benzene luekopenic rabbit," there is marked decrease in the reduced glutathione of blood and bone marrow. The interpretation of this finding is somewhat speculative, and the question arises as to whether the decrease followed or preceded the leukopenic state. Evidence indicates that the glutathione reduction precedes the leukopenia, for in one of the animals, the glutathione was almost absent, yet the leukocyte count was over 3000. Furthermore, there is no constant parallel between the glutathione and leukocytic variations. It is impossible, at this time, without further work, to evaluate the full significance of these findings and to do so would be indulging in mere speculation. The result indicates that the leukopenic effect of benzene may take place by depletion of the normal acceleration factor for cell division in the bone marrow, which appears to be reduced glutathione.

GLUTATHIONE IN VARIOUS HEMATOLOGIC STATES IN MAN

In comparison with the vast amount of work reported on the general subject of glutathione metabolism, determinations on human blood have been relatively infrequent, and those reported, have been carried out on a wide variety of clinical conditions. According to Heard,[16] after a study of sixteen normal subjects, the reduced glutathione in the blood ranges from 27 to 45 mgm./per cent, but in her report, no quotients are given. The various estimations for glutathione in normal blood can be seen in table 4, and it will be noted that the figures are fairly consistent, the average range being between 25 and 40 mgm./per cent. The estimations on normal blood that we have carried out, also fall within this range. The figures given in the literature, however, have little value for comparative purposes, since quotients are given in very few instances, and, therefore, the normal quotient for glutathione has not been well established. Woodward and Fry found Gabbe's quotient to range between 4.9 to 9.1, in a

series of twenty-three examinations and in our studies, we have found it to be between 5.7 and 8.6.

In spite of the large volume of work on glutathione changes in various diseases, there are very few reports concerning the blood dyscrasias, particularly those involving marked changes in the leukocytes. A summary of such studies is presented in table 5.

A study of these reports indicate that certain changes are found consistently in the few cases of pernicious anemia studied, these being mainly an increased or normal reduced glutathione with a high quotient, if determinations are made during relapse, while in remission both figures are at the normal level. However, in the hypochromic anemias, the glutathione is either normal or lowered, and the quotient is usually below normal.

We can find reports of glutathione studies in only three cases of leukemia, one of these being myeloid and two lymphatic, in each of which the cell counts were high. The glutathione was markedly elevated in the myeloid type, while definitely lowered in the lymphatic types, but unfortunately, no quotients were given in any of these cases.

In view of the paucity of reports concerning blood dyscrasias, characterized by marked leukocytic changes, we have studied the glutathione changes in twenty patients exhibiting a variety of hematologic states, including six cases of neutrophilic leukocytosis, associated with infections. The results obtained are presented in detail in table 5. In the one case of pernicious anemia, the reduced quotient was found to be markedly increased when the patient was in profound relapse, but was found to be normal at the peak of reticulocytosis. This is in accord with the findings of other investigators.

In a consideration of the findings in the twenty cases, we are impressed by the glutathione changes in three groups of blood disorders. In the first group, which consists of the myeloid leukemic states, there is a marked increase in the reduced quotient. We would describe this group as one characterized by a pathological leukocytic activity of the bone marrow. In the second group, which consists of those disorders in which there is apparent suppression or decrease in bone marrow activity, consisting of aplastic

TABLE 4

BLOOD GLUTATHIONE IN NORMAL AND ABNORMAL HEMATOLOGIC STATES, ACCORDING TO REPORTED CASES

AUTHORS	DIAGNOSIS	CASES	REDUCED GLUTATHIONE OF BLOOD	REDUCED QUOTIENT	REMARKS
Varela, K. et al.[35]	Normal		40–56		
Bowman[5]	Normal	93	27.5		
Schelling[34]	Normal (males)	10	21.3		
	Normal (females)	11	22.7		
Benedict and Gottschall[2]	Normal	11	23–35		By another method on same subjects, glutathione 29 to 42
Villela and Campos[38]	Normal (males)	15	27		Total glutathione 33.6
	Normal (females)	15	28.4		Total glutathione 36.2
Gabbe[10]	Normal		24–46		
Pickard and Marsden[23]	Normal	8	24.1		
Achard et al.[1]	Normal		35–50		
Heard[16]	Normal	16	27–45		
Varela, K. et al.[35]	Pernicious anemia	1	45		After treatment with liver, 32.0
	Hypochromic anemia	7	32		
Villela and Campos[38]	Polycythemia	1	55.8		Total glutathione 66.5. Erythrocytes, 10 million
Varela, B. et al.[37]	Hypochromic anemia	6	29.3		One case
	Pernicious anemia	5	26.4		One case after treatment 27, 29, 37
Litarczek et al.[26]	Lymphatic leukemia	1	16.8		
	Hypochromic anemia	1	14.1		
	Pernicious anemia	2	13.0		After treatment, one case from 11.8 to 14.1

Gabbe[10]	Pernicious anemia		N.S.	13.8–15.7	
	Hemorrhagic anemia		N.S.	5.3– 9.3	
	Secondary anemia		N.S.	9.3– 9.8	
Campanacci[7]	Secondary anemia	4	34	9.4	
	Pernicious anemia (relapse)	5	26.1	14.8	
	Pernicious anemia (remission)	3	21.2	5.0	
Platt[29]	Myelogenous leukemia	1	82.0		Leukocytes, 576,000
	Lymphatic leukemia	1	Low		
Pickard and Marsden[28]	Secondary anemia	23	24.9		Non-hemorrhagic
	Post hemorrhage anemia	11	17.1		
	Pernicious anemia	6	16.5		
	Pernicious anemia	1	34.2		After treatment (remission)

TABLE 5

BLOOD GLUTATHIONE IN VARIOUS BLOOD DYSCRASIAS AND IN PATIENTS WITH LEUKOCYTOSIS

CASE	DIAGNOSIS	BLOOD GLUTATHIONE				LEUKOCYTES	ERYTHROCYTES	REMARKS
		R.G.	R.Q.	T.G.	T.Q.			
1	Aleukemia, myeloid	13.5	16.2	16.8	20.3	4,850	830,000	
2	Leukemia, acute myeloblastic	18.7	17.0	20.4	18.5	15,000	1,100,000	
3	Agranulocytosis	15.5	3.8	33.4	8.1	1,350	4,100,000	No granulocytes
	Agranulocytosis	37.7	9.2	43.4	10.5	35,000	4,100,000	One week later after liver therapy
4	Aplastic anemia	11.9	4.5	23.9	9.0	6,250	2,660,000	
5	Idiopathic thrombopenia	26.4	5.0	31.3	5.9	6,350	5,230,000	
6	Aplastic anemia	7.3	5.8	16.8	13.4	650	1,200,000	
7	Chronic myeloid leukemia	23.6	5.9	24.5	6.1	50,000	4,000,000	
8	Lymphatic leukemia	18.6	9.3			30,000	2,000,000	
9	Chronic myeloid leukemia	51.8	12.3	56.0	13.3	140,000	4,210,000	
10	Polycythemia vera	43.6	4.6	71.2	7.5	20,000	9,400,000	
11	Pernicious anemia (relapse)	7.3	13.3	17.8	32.3	8,000	550,000	
	Pernicious anemia	11.9	5.9	22.4	11.2	7,300	2,000,000	One week later, after liver therapy. Reticulocytes 38 per cent
12	Hemolytic jaundice	34.6	8.8	47.2	12.1	5,750	3,900,000	
13	Achlorhydric microcytic anemia	14.7	7.1	32.2	15.4	13,400	2,090,000	
14	Sickle cell anemia	6.1	3.3	31.8	17.3	22,600	1,840,000	
15	Leukocytosis	37.1	8.5	49.7	11.5	20,750	4,330,000	Rectal abscess
16	Leukocytosis	28.8	5.8	36.5	7.4	14,650	4,960,000	Lobar pneumonia
17	Leukocytosis	35.6	6.6	43.3	8.0	19,050	5,380,000	Acute appendicitis
18	Leukocytosis	27.0	6.0	40.8	9.0	17,250	4,520,000	Cystitis
19	Leukocytosis	41.1	8.8	47.2	10.0	14,900	4,680,000	Pelvic inflammatory
20	Leukocytosis	21.0	5.3	39.6	10.0	18,850	4,000,000	Lobar pneumonia

anemia and agranulocytosis, there is a marked and definite decrease in the reduced quotient. In this connection, case 3 is of particular interest since the glutathione became normal after resumption of normal bone marrow activity.

In the third group, which shows increased physiological activity of the bone marrow, as illustrated by the six cases of neutrophilic leukocytosis associated with various infections, there was little deviation of the reduced quotient from the normal.

In the one case of lymphatic leukemia, the quotient was normal, in contrast to the myeloid types showing similar increases in number of white cells, in which the quotient was quite high. Similar findings have been observed in the other reported lymphatic leukemias. It would appear that the glutathione is increased mainly in those leukemias in which the bone marrow is concerned. This finding, coupled with the glutathione decrease in bone marrow aplasia, and the lowered quotient in benzene leukopenic animals, would suggest a possible relationship of importance. Furthermore, it should be emphasized that the simple increase in the number of leukocytes in the blood stream bears no relation to the reduced quotient. This is illustrated by case 2, an acute myeloid leukemia, with a leukocyte count of 15,000 and a reduced quotient of 17; and case 19, with a physiological leukocytosis of 14,900 and a reduced quotient of 8.8. Similar comparisons may be drawn from other cases shown in table 5.

The number of estimations on other disorders in the series is so small that any discussion or interpretation of the findings, is hardly warranted, except to point out the absence of positive findings of significance.

It will be noted that case 3 is one of agranulocytosis, with a leukocyte count of 1350, and complete absence of granulocytes. At which time the reduced quotient was very low. This patient was given injections of liver extract, intramuscularly, twice daily, for six days, with no other treatment, during which time the leukocyte count steadily rose to 35,000 with 95 per cent granulocytes, at which time the second determination of glutathione was made and the quotient was found to be entirely normal. Omission of therapy at that time has resulted in a gradual decrease of the

leukocyte count to the normal level, where it has remained since, with the patient in good health.

We have seen a negro woman in whom the diagnosis of aplastic anemia was made, and of such severity, that no improvement had resulted from sixteen transfusions. Several months later intensive therapy with injections of liver extract resulted in a return of the blood picture to normal. These instances led us to a consideration of the glutathione content of liver extract. We have now carried out determinations on five different such preparations and have found that reduced glutathione is present in large amounts, averaging slightly under 1000 mgm./per cent. This might suggest that perhaps a part of its therapeutic efficacy in the leukopenic states is due to its glutathione content. For purposes of comparison we have also determined the glutathione content of several batches of pentnucleotide, but find only a trace in this product.

The observations recorded here suggest a relationship of glutathione metabolism to the various hematologic disorders, and since glutathione has been shown to be an accelerator of cell division, such a relationship should be of considerable importance.

SUMMARY

(1) Since experimental agranulocytosis can be consistently produced in animals with benzene and some of its oxidation products, a consideration of a possible mechanism is discussed.

(2) A resumé of the accelerating action of glutathione on the rate of cell division suggests the possibility of this substance influencing bone marrow activity and further suggests that its depletion would lead to bone marrow aplasia with resulting leukopenic states.

(3) Experiments are presented in which agranulocytosis was produced in rabbits by the daily injection of benzene, with the leukopenic animals showing a marked depletion of the biologically active or reduced glutathione in bone marrow and blood.

(4) The glutathione changes in the blood of twenty patients with various hematologic disorders, showed a marked increase in those cases characterized by pathologic leukocytic stimulation;

a marked decrease in those showing bone marrow aplasia; and with no change in those showing physiologic leukocytosis.

(5) It is suggested that the reduced form of glutathione plays an important rôle in regulating normal bone marrow activity, and that depletion of this substance in the blood stream or bone marrow may lead to various leukopenic states.

(6) Determinations carried out on various preparations of liver extract show the presence of a large amount of reduced glutathione, suggesting that this substance may be partially responsible for the therapeutic effect of liver extract in some of the leukopenic diseases.

DISCUSSION

DR. S. P. REIMAN: I am glad to hear that distinctions are made between the proliferative, the differentiating and the organizing phases of cellular activity for, while they are dependent upon one another, yet they are distinct and separate biological processes subserving different ends, and arriving there by different morphological and, unquestionably also, chemical means.

Faced with the prospect of working with glutathione some years ago, our chemists chose the sulphur-containing moiety of this compound, namely, cystine, because of less chemical complications. They have found, however, that cystine itself is sufficient of a task. However, the addition of glutamic acid and glycine into the molecule to make glutathione, modified the chemical effect, as Hammett has shown, viz., that cystine stimulates proliferation, that glutamic acid accelerates differentiation and that glycine accelerates protein reconstitution (Science, **79:** 457. 1934).

In addition to the effect that the sulphydryl group has on cell proliferation as brought out by the evidence here presented, cystine also has an effect on blood coagulation in that it increases the length of time taken by these processes. This is also worthy of further study. I think I may safely take this work as another example of the profound biological influence of sulphydryl.

REFERENCES

(1) ACHARD, CH., LÉVY, J., AND GUTHMANN, M. G.: In Chem. abstr., **27:** 4580. 1933.

(2) BENEDICT, S. R., AND GOTTSCHALL, G.: The analysis of whole blood. IV. The determination of glutathione. Jour. Biol. Chem., **99:** 729–740. 1933.

(3) BINET, L., AND ARNAUDET, A.: Variations quantitatives du glutathion réduit dans le foie, suivant diverses conditions expérimentales. C. R. Soc. de Biol., **108:** 1117–1118. 1931.

(4) BLAKE, F. G., WINTERNITZ, M. C., GEIGER, A. J., LEONARD, J. C., AND OPPER, L.: Agranulocytic angina. Yale Jour. Biol. and Med., **7:** 465–471. 1935.

(5) BOWMAN, R. O.: Glutathione content of blood. Proc. Soc. Exper. Biol. and Med., **31:** 616–617. 1934.

(6) BROWN, H., AND KOLMER, J. A.: The relation of arsenicals to the glutathione content of animal tissues. Jour. Pharm. and Exper. Ther., **35:** 417–424. 1929.

(7) CAMPANACCI, D.: Kohlehydratstoffwechsel und lösliche Schwefelverbindungen in den Erythrocyten und Geweben. Klin. Wnschr., **9:** 1212–1213. 1930.

(8) CUSTER, R. P.: Studies on the structure and function of bone marrow. IV. Bone marrow in agranulocytosis. Am. Jour. Med. Sci., **189:** 507–515. 1935.

(9) deRey Pailhade: Quoted from BODANSKY, M.: Introduction to physiological chemistry. New York: John Wiley and Sons, p. 197. 1927.

(10) GABBE, E.: Über Vorkommer und Bedeutung lödlicher Schwefelverbindungen in den Blutkörperchen. Klin. Wnschr., **8:** 2077–2081. 1929.

(11) HAMMETT, F. S.: The natural chemical equilibrium regulative of growth by increase in cell number. Protoplasma, **11:** 382–411. 1930.

(12) HAMMETT, F. S., AND HAMMETT, D. W.: The influence of sulfhydryl and sulfoxide on differential growth within the regenerating chela of the hermit crab (Pagurus longicarpus). Protoplasma, **16:** 253–286. 1932.

(13) HAMMETT, F. S., AND HAMMETT, D. W.: The influence of sulfhydryl on the formation of aberrant disorganized overgrowths in the regenerating right chela of the hermit crab (Pagurus longicarpus). Protoplasma, **17:** 321–358. 1932.

(14) HAMMETT, D. W., AND HAMMETT, F. S.: Crucial demonstration of the proliferative growth regulating property of the naturally occurring chemical equilibrium composed of sulfhydryl and its partially oxidized derivatives. Protoplasma, **19:** 161–169. 1933.

(15) HARRISON, D. C.: The autocatalylic oxidation of sulphydryl compounds. Jour. Biochem., **21:** 1404–1415. 1927.

(16) HEARD, E. V.: Sulphur containing compounds in the blood. Unpublished.

(17) HOLDEN, H. F.: A note on the presence of glutathione in the corpuscles of mammalian blood. Jour. Biochem., **19:** 727–728. 1925.

(18) HOPKINS, F. G.: On an autoxidisable constituent of the cell. Jour. Biochem., **15:** 286–305. 1921.

(19) HONGO, S. In Chem. Abst., **26:** 3830. 1932.

(20) HUNTER, G., AND EAGLES, B. A.: Glutathione. A critical study. Jour. Biol. Chem., **72:** 147–175. 1927.

(21) KENDALL, E. C., AND NORD, F. F.: Reversible oxidation-reduction systems of cysteine-cystine and reduced and oxidized glutathione. Jour. Biol. Chem., **69:** 295–337. 1926.

(22) KRACKE, R. R.: The experimental production of agranulocytosis. Am. Jour. Clin. Path., **2:** 11–30. 1932.

(23) KRACKE, R. R., AND PARKER, F. P.: The etiology of granulopenia (agranulocytosis), with particular reference to drugs containing the benzene ring. Jour. Lab. and Clin. Med., **19:** 799–818. 1934.

(24) KRACKE, R. R., AND PARKER, F. P.: The etiology of granulopenia (agranulocytosis), with particular reference to drugs containing the benzene ring. Am. Jour. Clin. Path., **4:** 453–469. 1934.

(25) KUSHIYAMA, I.: In Chem. Abst., **28:** 3134. 1934.

(26) LITAROZEK, G., DINISCHIOTU, G., AND NESTORESCU, B.: Sur les variations du pourcentage en glutathion réduit dans divers états anémiques. C. R. Soc. D. Biol., **111:** 198–200. 1932.

(27) MATSUMORI, T., AND OKUDA, M.: Über den Glutathiongehalt des Muskels, bezw. der anderen Organe und Gewebe, besonders beim Kaninchen. Jour. Biochem., **11:** 407–414. 1930.

(28) PICKARD, R. J., AND MARSDEN, C. S., JR.: The relation of blood glutathione to the hemoglobin and amount of red cells. Jour. Lab. and Clin. Med., **19:** 395–404. 1934.

(29) PLATT, R.: The blood glutathione in disease. Brit. Jour. Exper. Path., **12:** 139–143. 1931.

(30) REIMANN, S. P., AND HAMMETT, F. S.: Cell proliferation response to sulphydryl in man. Proc. Soc. Exper. Biol. and Med., **27:** 20–22. 1929.

(31) REIMANN, S. P.: Use and reasons for the use of thiocresol to stimulate wound healing. Jour. Am. Med. Assoc., **94:** 1369–1371. 1930.

(32) REIMANN, S. P.: The hyperplastic reaction of the skin to sulphydryl and its significance in neoplasia. Am. Jour. Cancer., **15:** 2149–2168. 1931.

(33) REIMANN, S. P.: Aberrant growths in regenerating claws of the hermit crab under the influence of sulphydryl. Their relation to neoplasia. Arch. Path., **15:** 675–697. 1933.

(34) SCHELLING, V.: A study of the blood glutathione. Jour. Biol. Chem., **96:** 17–24. 1932.

(35) VARELA, K., APOLO, E., AND VILAR, A.: Das Glutathion im Blute bei, pathologischen Zuständen. Klin. Wchnschr., **9:** 1029–1030. 1930.

(36) VARELA, B., DUOMARCO, J., AND MUNILLA, A.: Modifications du glutathion réduit du sang et des organes dans l'anémie expérimentale produite par des aignées répétées chez le chien et le lapin. C. R. Soc. d. Biol., **106:** 837–839. 1930.

(37) VARELA, B., DUOMARCO, J., AND VILAR, J.: Modifications du glutathion réduit du sang dans les anémies humaines. C. R. Soc. d. Biol. **106:** 835–837. 1931.

(38) VILLELA, G. G., AND CAMPOS, J. G.: Glutathion du sang humain normal. C. R. Soc. d. Biol., **115:** 83–84. 1934.

(39) WOODWARD, G. E., AND FRY, E. G.: The determination of blood glutathione. Jour. Biol. Chem., **97:** 465–482. 1932.

BLOOD PHOSPHATASE: ITS CLINICAL SIGNIFICANCE*

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Until recently very little was actually known about the enzyme activity of the blood and particularly about its phosphatase activity. While Bloor[1] in 1918 reported an increase in the inorganic phosphates of the blood on standing for several hours after withdrawal, no especial value was placed on this observation and its significance was lost until about 1923. At that time Robison,[12] while studying the hydrolysis of phosphoric esters by various enzymes, made several observations which shed light on the metabolism of calcium and phosphorus and lead him and other investigators to undertake research which explained the observation of Bloor. These investigations showed that the increase of inorganic phosphates in the blood on standing was due to a phosphatase hydrolysis of the phosphoric esters of the plasma.

Robison,[13] in his studies on phosphoric ester hydrolysis, was especially struck by the occurrence of a precipitate of insoluble calcium phosphate during the hydrolysis. This observation suggested to him the possibility that a similar mechanism might be concerned with the deposition of calcium salts in bone. As a result of their investigations, Robison[13] and his co-workers advanced the hypothesis that the osteoblasts and hypertrophic cartilage cells in the periosteum and epiphyseal zones of the bone secrete an enzyme which hydrolyzes the phosphoric esters of the blood and brings about a local increase in the concentration of phosphate ions with a deposition of calcium phosphate in the ossifying zone.

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Kay[9] summarized the evidence in favor of this view and concluded that

> in considering problems of bone formation, maintenance and pathology an active phosphatase in the epiphyseal zone and periosteum can no longer be ignored but must be accepted as playing a very important rôle in these processes.

Kay[9] also demonstrated an increase of plasma phosphatase in certain generalized diseases of bone and suggested that this increase in plasma phosphatase is due to a leakage of the enzyme out of the bone into the plasma.

Robison[13] thought the source of the plasma phosphatase under normal conditions may be due to a normal breakdown of the cellular elements of the blood which have been shown to contain an enzyme capable of hydrolyzing phosphoric esters. Bodansky and Jaffe[3] believed that the serum phosphatase is probably of diverse origin and that its increase or decrease is caused by stimulation or suppression of the activities of the specific tissues in which it is found. This view seems logical as increases in serum or plasma phosphatase have been demonstrated in conditions other than bone disease.

Enzymes capable of hydrolyzing phosphoric esters have been shown to exist in large amounts in the intestinal mucosa and in the kidney,[7] in addition to the epiphyseal zone and periosteum of the bone. They have also been found in smaller amounts in the liver, spleen, pancreas and muscles. The function of these phosphoric ester hydrolyzing enzymes has been the subject of much discussion and it is probable that the phosphatase in the intestinal mucosa plays an important part in the hydrolysis of the phosphoric esters of the food,[7] and that of the kidney in the excretion of inorganic phosphorus.[5]

The determination of serum or plasma phosphatase by the methods available at the present time is not a direct measure of the enzyme but rather a measure of its activity in hydrolyzing a substrate of phosphoric esters. During this hydrolysis there is set free inorganic phosphate which can be accurately estimated before and after hydrolysis under standard conditions. The earlier methods of determining the phosphatase activity of the plasma were too cumbersome for routine clinical laboratories,

requiring as they did large amounts of plasma or serum, long periods of hydrolysis, and accurate hydrogen ion checks. Within the past few years however there have appeared in the literature several methods which have tended towards simplification of this technic. Unfortunately, however, there has been no unanimity of thought in setting up a standard unit of phosphatase activity so that each author adopts his own unit calculated on a set of conditions which do not prevail in the other methods. This results in confusion when the figures obtained by one method are compared with those obtained by another.

To determine which method was most suitable for adoption by a clinical laboratory, both because of simplicity of technic, time consumed and consistency of results, three of the more recent methods were used simultaneously on a series of bloods from normal controls, patients with bone pathology and patients with no bone pathology but with other pathologic processes.

(1) The Jenner and Kay technic.[8] These authors have modified the original Kay technic so that while this technic is slightly less precise than the original method, it is more practical and suitable for clinical work. The modifications consist of a reduction of the time of hydrolysis from five to three hours; reduction of the plasma required from 5 cc. to 2 cc.; and the elimination of filtration of the centrifuged plasma, and hydrogen ion determinations. In spite of these modifications the method is still quite cumbersome. The incubation period of three hours is long and the reagents rather complex for routine clinical work. In addition to the technical disadvantages, the amount of plasma used in the test (0.25 cc. in each tube) is too small to give constant results.

(2) Bodansky technic.[2] Bodansky improved upon the Jenner-Kay technic and claims additional accuracy and convenience by (a) using serum instead of oxalated plasma which retards slightly phosphatase activity; (b) by corrections for analytical errors in the presence of glycerophosphate and trichloracetic acid; (c) by the use of a combined solution of buffer and substrate of good keeping quality; (d) by the reduction of the time of hydrolysis to one hour. This method considered from the standpoint of clinical adaptability seems to be the best method available at present. The time consumed is not too long and the results are consistent. The one disadvantage is the need of at least 4 cc. of serum, but this is not a serious obstacle.

(3) King technic.[10] In this method the author determines the phosphatase activity by measuring the amount of phenol liberated by the action of serum phosphatase on a substrate of di-sodium phenyl phosphate. King accomplishes his object of elaborating a more rapid technic by reducing the time of hydrolysis

to one half hour. In order to do this and at the same time express his results in units numerically equal to that of Jenner and Kay, he takes advantage of the fact that di-sodium phenyl phosphate is hydrolyzed at double the rate of di-sodium B glycerophosphate and that approximately three times as much phenol by weight is liberated as phosphorus. The method is basically sound, good color is developed in the hydrolyzed tubes, but very little color in the control tubes. This great difference of color, which makes for difficult matching, constitutes its chief disadvantage.

In general, there is an approximate similarity of the Jenner and Kay and the King units, both of whose values are about three times that of the Bodansky unit.

The results of this study would indicate that the determination of the serum or plasma phosphatase is of definite value in the differential diagnosis of certain conditions and in general agree with

TABLE 1
NORMAL VALUES

CASE	AGE	SEX	PHOSPHATASE UNITS		
			Bodansky	Jenner-Kay	King
	years				
1	12	F.	4.0	14.6	17.1
2	13	M.	4.0	14.6	18.8
3	6	F.	3.8	15.3	17.5
4	58	F.	2.7	12.2	13.2
5	26	M.	1.2	9.1	7.2
6	27	M.	2.6	11.6	9.7
7	26	M.	1.9	10.1	8.9
8	30	M.	2.7		13.6
9	61	M.	2.1		
10	10 mos.	M.	4.3		

the findings of other investigators. While the volume of published clinical data is much too small to draw definite inferences as to the complete scope of the value of blood phosphatase determinations, nevertheless there are several conditions in which there has been shown a consistent and definite increase of this enzyme in the blood.

In a small series of determinations on normals to check our technic, the amounts of phosphatase in the plasma or serum was found to conform to the published normals which are given for adults by Bodansky as 1.5 to 4.0 units, by Jenner-Kay as 3.2 to 7.9 units, and by King as 3.7 to 13.1 units. Bodansky gives the value for children as 3.1 to 13.1 units.

In Paget's disease with multiple bone involvement the phosphatase activity of the blood serum may rise to twenty or more times the normal figures. The cases of Paget's disease presented in this paper do not show quite so marked a rise but this may be explained by the fact that these cases, when seen, showed definite evidence of healing with beginning sclerosis of the involved bones. In localized bone pathology, while a normal serum phosphatase does not rule out Paget's disease, a high blood phosphatase will strengthen the evidence in favor of a diagnosis of this condition.

TABLE 2

GENERALIZED BONE DISEASE

CASE	AGE	SEX	PHOSPHATASE UNITS, BODANSKY	DIAGNOSIS
	years			
1	59	M.	24.1	Polyostotic Paget's disease
2	55	M.	21.7	Polyostotic Paget's disease
3	45?	F.	12.2	Polyostotic Paget's disease
4	48	F.	10.1	Localized Paget's disease
5	1½	M.	16.0	Rickets in stage of healing
6	1¼	M.	4.9	Osteogenesis imperfecta
7	19	F.	2.2	Osteogenesis imperfecta
8	12	M.	3.9	Osteogenesis imperfecta
9	46	F.	2.0	Multiple myeloma
10	23	M.	3.1	Multiple destructive neoplastic bone lesions, central in origin (multiple myeloma)
11	2	F.	2.9	Erythroblastic anemia (Cooley)
12	42	F.	8.0	Chronic myelogenous leukemia
13	44	F.	4.3	Multiple bone metastasis. Carcinoma of adrenal

One case of this series (case 4, table 2) was diagnosed as metastatic carcinoma of bone but showed an increased phosphatase. Later developments showed the localized bone condition to be Paget's disease and not of metastatic origin. Occasionally difficulty arises in the differential diagnosis of Paget's disease and the bony changes occurring in hyperparathyroidism. While some slight increase in blood phosphatase may occur in hyperparathyroidism, it is never as marked as in Paget's disease.

Case 2,* table 2, was diagnosed roentgenologically and clinically

* This case was studied through the courtesy of Dr. Joseph Echikson.

as hyperparathyroidism, but because of the high blood phosphatase figure was restudied and definitely shown to be a case of polyostotic Paget's disease.

Although only one case of rickets occurred in this series, it is probable that in this condition the determination of the phosphatase activity of the blood will be extremely important both in confirming the diagnosis and in following the effect of treatment.

Smith[14] has shown that the blood phosphatase is increased in active rickets, the degree corresponding to the severity of the disease. He has also demonstrated that the increase in phosphatase occurs early in the disease while x-ray does not show its presence until the condition is fairly well advanced. In follow-

TABLE 3

PATHOLOGIC CONDITIONS NOT INVOLVING BONE PATHOLOGY

CASE	AGE	SEX	PHOSPHATASE UNITS			DIAGNOSIS
			Bodansky	Jenner-Kay	King	
	years					
1	48	F.	7.1		19.3	Obstructive jaundice
2	44	F.	13.4	23.4	40.0	Obstructive jaundice
3	35	M.	8.6			Obstructive jaundice
4	34	F.	2.9			Multiple xanthomatosis of tendons
5	56	F.	6.7		19.3	Diabetes mellitus
6	65	M.	5.6		20.6	Gout
7	34	F.	3.9		13.7	Subacute bacterial endocarditis

ing the effect of treatment, Smith and Maizels[15] showed that while the calcium and phosphorus of the blood returned to normal within two to three weeks after commencement of treatment, the phosphatase activity of the blood declined more gradually and did not reach normal until the healing process was complete.

In obstructive jaundice, Roberts[11] has shown that the blood phosphatase figure is invariably high, whereas in all other varieties of jaundice the figure is either slightly raised or may be entirely normal, although the patient be deeply jaundiced. He felt that the phosphatase reaction can be used to differentiate obstructive from toxic jaundice. In the three cases in this series

(cases 1, 2 and 3, table 3) the jaundice was of the obstructive type and the blood showed an increase in phosphatase activity. No cases of non-obstructive jaundice were available for study. It is interesting to note the apparent lack of relationship between the phosphatase activity and the amount of bilirubin in the blood. Bodansky and Jaffe[4] suggest the possibility that they represent disturbances of different functions of the liver.

TABLE 4

FRACTURES AND INFECTION OF BONE

CASE	AGE	SEX	PHOSPHATASE UNITS			DIAGNOSIS
			Bodansky	Jenner-Kay	King	
	years					
1	58	M.	5.0	13.1	17.1	Fracture os calcis—2 days
2	43	M.	5.0	9.5	12.3	Fracture radius and ulna—6 days
3	49	M.	6.6	14.7	21.2	Fracture tibia and fibula—1 week
4	35	F.	5.2	9.9	16.6	Fracture radius and ulna—12 days
5	54	F.	6.7	14.5	24.2	Fracture femur—12 days
6	40	F.	6.0	12.4	15.8	Fracture tibia and fibula—4 weeks
7	50	F.	4.7	9.4	14.2	Fracture jaw—5 weeks
8	37	M.	3.9	9.2	7.3	Fracture radius and vertebra—7 weeks
9	74	F.	8.1	15.1	28.1	Fracture tibia, fibula, elbow and pelvis—7 weeks
10	43	M.	9.4	18.9	16.8	Fracture tibia and fibula—6 months Sliding graft—7 weeks
11	37	M.	2.3			Destructive osteoarthritis
12	80	M.	3.9			Osteoarthritis
13	31	M.	5.0		16.2	Infectious arthritis left hip joint
14	21	M.	3.3		9.6	Chronic infectious arthritis-poly-articular
15	63	M.	2.4			Osteomyelitis of jaw
16	37	F.	7.8	19.3	24.2	Tuberculous hip joint
17	26	M.	3.6		17.2	Osteomyelitis of femur-sepsis

Several other conditions showing multiple bone pathology occurred in this series and are of differential diagnostic significance. In three cases of osteogenesis imperfecta, two of which showed typical blue sclera, the blood phosphatase activity was normal. In a case of erythroblastic anemia with marked changes in the entire skeletal system, and in two cases of multiple myeloma,

the blood phosphatase activity was within normal limits. In this series of fractures the values in Bodansky units ranged from 3.9 to 9.4, an average of 6.0, which is somewhat above the high normal limits for adults. Hunsberger and Ferguson[6] also observed an increase in phosphatase activity of the blood in fractures.

The results shown in table 4 do not apparently indicate any relationship between the amount of phosphatase and the time elapsed since the fracture was sustained but seems to point to some relationship to the severity of the injury, the highest figures (cases 9 and 10) showing multiple fractures.

Smith and Maizels however found an increase in phosphatase beginning about ten days after the fracture which is maintained until healing is complete, the elevation of phosphatase corresponding to the amount of callus laid down.

Arthritis (table 4) apparently does not cause an increase in blood phosphatase.

SUMMARY AND CONCLUSIONS

Methods for the determination of blood phosphatase activity have recently been made available. The phosphatase activity of the blood is of clinical significance in pathologic changes in phosphatase rich tissues and especially in bone. A comparison of three recent methods is presented together with the findings in a series of cases of bone disease and diseases of other systems. Determination of blood phosphatase activity has been shown to be of definite value in the diagnosis of Paget's disease, active rickets and obstructive jaundice and of aid in following the results of treatment in these conditions.

REFERENCES

(1) Bloor, W. R.: The distribution of phosphoric acid in normal human blood. Jour. Biol. Chem., **36:** 49–57. 1918.

(2) Bodansky, A.: Phosphatase studies. II. Determination of serum phosphatase, factors influencing the accuracy of the determination. Jour. Biol. Chem., **101:** 93–104. 1933.

(3) Bodansky, A., and Jaffe, H. L.: Phosphatase studies. Significance of clinical and experimental serum phosphatase variations; their osseous and non-osseous origins. Jour. Biol. Chem., **105:** xi–xii. 1934.

(4) Bodansky, A., and Jaffe, H. L.: Phosphatase studies. VIII. Increase of serum phosphatase after bile duct ligation in dog. Proc. Soc. Exper. Biol. and Med., **31:** 1179–1181. 1934.

(5) Eichholtz, F., Robison, R., and Brull, L.: Hydrolysis of phosphoric esters by the kidney in vivo. Proc. Roy. Soc., **99:** 91–106. 1925.

(6) Hunsberger, A., and Ferguson, L. K.: Variations in phosphatase and inorganic phosphorus in serum during fracture repair. Arch. Surg., **24:** 1052–1060. 1932.

(7) Hunter, D.: The significance to clinical medicine of studies in calcium and phosphorus metabolism. Lancet, **1:** 897–904. 1930.

(8) Jenner, H. D., and Kay, H. D.: Plasma phosphatase, III. A clinical method for the determination of plasma phosphatase. Brit. Jour. Exper. Path., **13:** 22–27. 1932.

(9) Kay, H. D.: Plasma phosphatase. Jour. Biol. Chem., **89:** 249–266. 1930.

(10) King, E. J., and Armstrong, A. R.: Convenient method for determining serum and bile phosphatase activity. Can. Med. Assn. Jour., **31:** 376–381. 1934.

(11) Roberts, W. M.: Blood phosphatase and the van den Bergh reaction in the differentiation of the several types of jaundice. Brit. Med. Jour., **1:** 734–738. 1933.

(12) Robison, R.: The possible significance of hexosephosphoric esters in ossification. Biochem. Jour., **17:** 286–293. 1923.

(13) Robison, R.: The significance of phosphoric esters in metabolism. New York: N. Y. Univ. Press, 1932, pp. 104.

(14) Smith, J.: Plasma phosphatase in rickets and other disorders of growth. Arch. Dis. Childhood, **8:** 215–220. 1933.

(15) Smith, J., and Maizels, M.: The plasma phosphatase in rickets and scurvy. Arch. Dis. Childhood, **7:** 149–158. 1932.

THE SIZE DISTRIBUTION OF LYMPHOCYTES IN HUMAN BLOOD FILMS*

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A great deal is known about the sizes and size variations of erythrocytes both in films (Price-Jones[5]) and in the unfixed state (Ponder[4]). Some of this information throws light upon the structure and function of erythrocytes, some of it is of great use in the diagnosis of disease and all that is of value has been obtained by the measurement of large numbers of cells. There is comparatively little exact knowledge regarding the sizes and size variations of lymphocytes of healthy persons, and the first object of this paper is to increase that knowledge. Its second purpose is to display the manner in which lymphocytes of different sizes are distributed in blood films, so as to test certain hypotheses regarding the causes of a now well recognized fact, namely that leukocytes of different classes (granular cells, lymphocytes and monocytes) are distributed in different proportions in different parts of blood films. This fact, which is of importance in the differential blood count, has been attributed by Gyllenswärd[2] to the differences in size of the different types of cells, whereas Silberstern[6] has claimed that it is due to differences in surface tension, associated with differences in agglutination properties possessed by the different types of leukocyte. The best test of these suggestions is to ascertain whether cells that are all of the same type are distributed in accordance with size in different parts of the film.

MATERIAL AND METHODS

The films used were prepared from drops of blood taken from the ears of two healthy male students, whose ages were, twenty-four and thirty years re-

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spectively. The drops were drawn along the slides by a square number 2 coverglass (22 mm. wide), held at an angle to the slide. The films were stained by Wright's stain and were chosen because they were thinly and evenly spread and well stained. They were mounted in balsam and covered. Their dimensions were approximately—22 x 30 mm. and 22 x 35 mm.

All measurements were made under immersion oil with a Zeiss filar screw eyepiece micrometer. For the principal film the objective was a Zeiss 90× lens (n.a. 1.30), and the linear magnification was about 1000. For the supplementary films the same micrometer was used with a Zeiss oil immersion objective 50 × (n.a. 0.85), giving a linear magnification of about 500. Most of the calculations were made on the micrometer readings, but the final results, that is means, were converted into micra by a factor obtained from a stage micrometer. The measurements taken on the cell bodies were: long axis, two readings; transverse axis (found by rotating the eyepiece through a right angle), two readings. Addition of these and division by four gave the mean diameter of the cell. On the principal film a complete survey of all the fields was carried out. The observations were begun at the top left-hand corner and the slide was moved up by the mechanical stage, field by field. Each eyepiece field, as it was reached, was recorded by the figure "1" on a sheet of paper in a typewriter, and, if lymphocytes were in the field, they were measured and a pencil mark for each lymphocyte was made near the "1" that represented that field. When the bottom of the film was reached, a move was made to the next adjacent field on the right and the film was then moved vertically again until the top was reached. Thus the film was surveyed in 172 vertical columns, each column being one field (about 0.16 mm.) wide. The typewritten marks representing the fields in columns formed a kind of chart of the film, and at the end of the survey it was possible to refer the measurements of the lymphocytes to their proper positions on the chart. This survey took several weeks, and throughout that time the microscope was touched only by the observer (B. K. C.).

At the beginning and end of the film there was naturally some irregularity and certain fields showed no blood cells, but, if these unoccupied areas had been omitted, it would have been impossible to place accurately on the chart the next cells met in the same column. Therefore, to make it possible for the observer to judge when he was passing from one field to the next, the top of the coverglass was faintly stained with Wright's stain, and the pattern of this staining was used in passing from field to field. The stain was so thin that it did not interfere with the definition and measurement of the cells. In order to divide the film, or rather its chart, into 64 rectangles, the 172 columns were grouped into eight groups of twenty-one or twenty-two columns each. The number of fields per column was on the average about 160, and each column was divided into eight equal parts. Thus sixty-four rectangles were obtained, which were, roughly, twenty-one or twenty-two fields wide and nineteen or twenty fields from above down. This subdivision is shown in table 1.

In order to compare the sizes of lymphocytes near the edges (upper and

lower) of the film with the sizes along the middle horizontal line, a cell was selected within the first six fields (about 0.9 mm.) of the top, and compared with a cell in the same vertical line near the middle of the film. This was done ten times at various points along the film, and a similar comparison was made between the middle and the lower edge of the film.

On the supplementary films the following observations were carried out, all the measurements being made by one observer (W. H.): (1) Four squares,

TABLE 1

CONDENSED CHART OF PRINCIPAL FILM, DIVIDED INTO SIXTY-FOUR RECTANGLES, SHOWING IN EACH RECTANGLE THE NUMBER OF LYMPHOCYTES (IN PARENTHESES) AND THE MEAN DIAMETER (IN μ) OF THE LYMPHOCYTES

UPPER EDGE OF FILM

	ROW-NUMBERS	COLUMN-NUMBERS								ROW-MEANS	
		I	II	III	IV	V	VI	VII	VIII		
BEGINNING (LEFT END) OF FILM	1	(11)	(17)	(25)	(27)	(19)	(16)	(16)	(16)		END (RIGHT END) OF FILM
		12.38	11.01	11.03	11.37	12.35	11.99	12.60	12.88	11.95	
	2	(12)	(30)	(49)	(59)	(26)	(17)	(19)	(30)		
		11.61	10.36	9.41	10.65	11.62	13.00	12.26	12.39	11.41	
	3	(17)	(41)	(80)	(58)	(31)	(15)	(18)	(20)		
		11.38	9.67	9.31	10.66	11.87	12.13	12.47	12.42	11.24	
	4	(18)	(37)	(40)	(39)	(13)	(11)	(9)	(13)		
		10.30	9.59	9.50	10.47	11.57	13.04	12.34	12.47	11.16	
	5	(5)	(21)	(57)	(42)	(26)	(20)	(27)	(21)		
		10.36	9.95	9.76	10.45	12.19	12.78	13.05	12.43	11.37	
	6	(3)	(27)	(43)	(25)	(15)	(12)	(15)	(19)		
		11.31	10.44	11.07	11.30	11.59	12.85	12.72	12.21	11.69	
	7	(2)	(30)	(39)	(31)	(13)	(19)	(17)	(30)		
		12.22	11.56	10.88	11.43	11.95	12.30	12.49	12.44	11.91	
	8	(3)	(18)	(22)	(13)	(11)	(16)	(9)	(29)		
		11.95	10.68	11.50	11.92	12.15	13.25	12.39	10.71	11.82	
	Means....	11.44	10.41	10.31	11.03	11.91	12.67	12.54	12.24		

LOWER EDGE OF FILM

each of 3 to 5 mm. side, were marked with ink on the upper surface of the coverglasses, at various points in a horizontal row through the middle of the film. In each square ten lymphocytes were measured (two measurements of long axis and two of transverse axis). (2) At various points within 1 mm. from the upper edge, ten lymphocytes were measured, and each of these was compared with the measurement of a lymphocyte in the same vertical line but situated near the middle of the film. A similar comparison was made between ten cells near the lower edge and ten cells near the middle of the film.

FREQUENCY DISTRIBUTION OF LYMPHOCYTE SIZES

The total 1658 lymphocytes in the principal film were grouped according to their mean diameters recorded in table 2.

The frequency polygon (fig. 1) is unimodal. There is no suggestion of two modes or peaks. This agrees with a survey, just completed, of the lymphocyte sizes in fifty-five patients with various diseases and some without disease (Mainland, Coady and Joseph[3]), but the present example is more definite because of

TABLE 2

MEAN DIAMETERS ON PRINCIPAL FILM

MEAN DIAMETER	NUMBER OF LYMPHOCYTES
μ	
4.5	
5.5	1
6.5	20
7.5	90
8.5	223
9.5	237
10.5	257
11.5	235
12.5	207
13.5	147
14.5	104
15.5	84
16.5	40
17.5	13
18.5	
	1658

the large number of cells examined in one film. There is, therefore, no justification for reporting on large and small lymphocytes in blood and films as if they belonged to two separate groups. In the study just referred to, there was also a strong indication of a skewness of the distribution; the tail on the right, representing large diameters, spread out farther from the mode than did the tail on the left. This indicated a much greater tendency for the occurrence of unusually large cells than for the occurrence of unusually small cells. With the large number of cells in the

present survey, it was possible to prove that the distribution differed significantly from the symmetrical normal curve. The tests applied were those of Fisher[1], and the results were:

$$g_1 = +0.36; \text{ standard error of } g_1 = \pm 0.061$$
$$g_2 = -0.52; \text{ standard error of } g_2 = \pm 0.121$$

Each of these values is more than four times its standard error, and therefore highly significant. The interpretation is as follows: g_1 is a measure of skewness, and, as g_1 is significant, it shows that

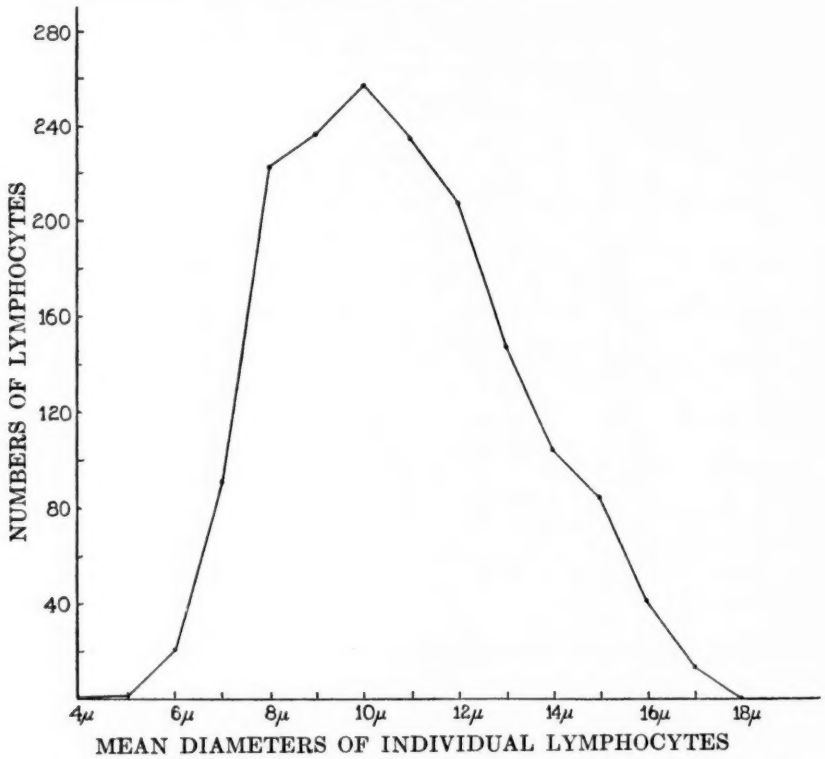

FIG. 1. FREQUENCY DISTRIBUTION OF SIZES OF LYMPHOCYTES IN PRINCIPAL FILM

the skewness of fig. 1 could not be adequately accounted for by random sampling of material in which the true distribution was normal in form. This result can be combined with the wider survey mentioned above, and the conclusion is that in blood films,

even where there is no indication of pathological influences on the lymphocytes, moderately small lymphocytes are more frequent than moderately large ones, and very large lymphocytes are more frequent than very small ones.

It will be noted that this statement refers to fixed and stained cells and it might be asked whether an originally normal distribution was changed to an asymmetrical one during treatment. Thus, Wiseman[7] found that lymphocytes in films had a greater average size than lymphocytes taken from the same blood but supravitally stained. If it is supposed that fixation and staining increase the cell diameters by about one quarter of their original size, then the large lymphocytes will be increased by more, in actual micra, than the small ones, and the right-hand (higher) end of the frequency curve will be pushed more to the right than is the left-hand (lower) end. If the original distribution is normal in shape, this will produce a skewness of the type seen in figure 1, but this does not, of course, prove that the suggested process is the actual cause, or that the original curve is symmetrical. It is beyond the scope of this paper to establish the form of the curve for living lymphocytes.

There is a striking contrast between lymphocytes and erythrocytes in respect of the frequency distribution of sizes, because the sizes of erythrocytes in films from healthy persons form frequency curves that agree closely with the normal (Price-Jones). Fixation affects these cells also, but it causes shrinkage instead of enlargement (Ponder). There is no obvious explanation for these differences between the erythrocytes and the lymphocytes.

The second of the measures mentioned above, g_2, being significant and negative, shows that, as compared with the normal curve, the shoulders of the lymphocyte size frequency curve are filled out at the expense of the summit and tails. This indicates that, relative to the total variation, there is a greater tendency for the occurrence of medium-sized cells than there is in a series of erythrocytes in films.

AVERAGE SIZES

The arithmetic average of the 1658 mean diameters was 11.24μ and the standard error of this average was $\pm 0.059\mu$. Ponder

points out that errors in determining the magnification or in converting micrometer readings to micra can introduce considerable discrepancy between different determinations of cell size. In the present investigation there is no certainty of accuracy beyond the statement of the manufacturers of the stage micrometer, a Zeiss instrument, used to calibrate the eyepiece micrometer. Hence there is hardly justification for doing more than stating that the mean diameter was of the order of 11μ. This vagueness does not, of course, vitiate the comparisons of lymphocyte size in different parts of the film or any of the conclusions regarding the shape of the frequency distribution or regarding relative variation, the coefficient of variation.

Wiseman's measurements of lymphocytes in films gave a modal diameter similar to the mean value in the present series. On the other hand, in the survey of fifty-five patients mentioned above, the average for the whole series was between 8 and 9μ, not much greater than the erythrocyte diameters. Although some individual patients had higher mean lymphocyte diameters than this, none was as high as 11μ. The difference from the present example cannot be accounted for by error in magnification. Moreover, in the series of fifty-five patients the erythrocytes were similar in size to those of healthy persons as determined by Price-Jones, nearly 8μ—another evidence that magnification is not responsible, because mere inspection showed that in the principal film in the present investigation the lymphocytes were on the average considerably larger than the erythrocytes. No final explanation of the difference between the two sets of observations can yet be given, but the following points may be noted: (1) In the films with the small lymphocyte sizes (from the fifty-five patients), measurements were made more in the central parts than at the periphery, and, as shown below, the lymphocytes in the middle tend to be smaller than at the ends or edges. (2) The films from the fifty-five patients were thicker than the principal film used here, and had been prepared by pushing the blood by a slide instead of a coverglass. The greater thickness of films lengthened the period in water necessary for differention. Investigations are now being carried on to determine the influence of different factors in the preparation of the film.

SIZE VARIATION

The variation between the sizes of the 1658 lymphocytes in the special film was represented by a standard deviation of $\pm 2.4075\mu$. This, expressed in terms of the mean, gave a coefficient of variation of 21.42 per cent—a high variation. The coefficients of variation found in the survey of 55 patients varied from 10 to 22 per cent, and for comparison it should be noted that Price-Jones, using the films from 100 healthy persons, found that the coefficients of variation for red cells varied between 5.64 and 7.26 per cent. Even if allowance is made for the possibility that the red cells measured were near the center of the films, and that outlying

TABLE 3

COMPARISON OF COLUMNS I AND II IN TABLE 1

ROW NUMBER	MEAN IN COLUMN I MINUS MEAN IN COLUMN II
	μ
1	+1.37
2	+1.25
3	+1.71
4	+0.71
5	+0.41
6	+0.87
7	+0.66
8	+1.27
Mean difference.....	+1.031

red cells might differ from those in the center, as do the lymphocytes (see below), this will hardly account for the great difference between the variation in lymphocytes and red cells. The present investigations cannot, of course, show whether this difference exists in the living condition or whether the size-reaction of lymphocytes to fixation and staining is more varied than the reaction of red cells.

SIZE DIFFERENCES IN DIFFERENT PARTS OF THE FILM

Table 1 shows the subdivision of the principal film into eight rows and eight columns, 64 rectangles, as described in the section

on material and methods. The numbers of cells* and the mean diameter is shown in each rectangle. Tests of the difference between the different rows and columns were made by Fisher's t test for the significance of a mean. Thus, in the comparison of columns I and II, (table 3) $t = 6.683$; $n = 7$; P is much less than 0.01.

The mean difference is highly significant, which indicates that the sizes in column I are greater than in column II, and that the difference is too great to be attributed to variations in random sampling. The same test was applied except in cases where the result was obvious, and the conclusions can be expressed as follows ("significant" indicating P between 0.05 and 0.01; "highly significant" indicating P less than 0.01):

Sizes in column I highly significantly greater than in column II

Sizes in column II not significantly different from those in column III

Sizes in column IV highly significantly greater than in column III

Sizes in column V highly significantly greater than in column IV

Sizes in column VI significantly greater than in column V

Sizes in column VII not significantly different from those in column VI

Sizes in column VIII not significantly different from those in column VII

Sizes in column VI highly significantly greater than in column I.

These results can be stated in more general terms thus: on the average, in the zone near the beginning of the film large lymphocytes were found; the smallest were between this zone and the middle. Thereafter the average sizes increased until the last quarter of the film was reached, but in that last section there was no appreciable change. The greatest average sizes in the second half of the film were greater than those near the beginning.

The supplementary films gave the following information, based on ten lymphocytes measured in each square, all the squares

* Some of the cells had to be omitted in constructing table 1, because of lack of certainty regarding their exact location.

being in a row running horizontally through the middle of the films:

Film 16R4

	Mean Diameter (μ)
Square I, near beginning of film	11.88
Square II, in first half of film	10.72
Square III, near middle of film	12.26
Square IV, near end of film	19.05

The t test, for comparison of means showed that the differences between the means in adjacent squares were all highly significant.

Film 16R2

	Mean Diameter (μ)
Square I, near beginning of film	8.47
Square II, in first quarter of film	7.44
Square III, in third quarter of film	8.21
Square IV, in last quarter of film	9.95

The difference between the means in I and II is significant (P being between 0.05 and 0.02); between II and III it is not significant; between III and IV it is highly significant.

The three films all agreed, therefore, in showing that larger lymphocytes were found near the beginning and also in the latter half of the film, with an intervening area of smaller lymphocytes.

In order to compare the parts of the film near the edges with parts in the interior, the means in row 2 of table 1 were subtracted from the means in row 1, and a similar comparison was made between row 8 and row 7. No significant differences were found. The outer rows, however, extended a considerable distance in from the edges, and therefore further comparisons were made. In the principal film there were not many lymphocytes near the edge, so that, within the first six fields (about 0.9 mm.) from the upper edge there were only ten lymphocytes to compare with cells in the same vertical line near the middle and there were only three lymphocytes within six fields of the lower edge. In each of the thirteen cases the size of the middle cell was subtracted from that of the corresponding cell near the edge. In three instances the difference was negative. The mean difference was $+2.12\ \mu$, which was proved significant by Fisher's t test for the significance of a mean (P between 0.05 and 0.02). In the supple-

mentary films, cells along the upper and lower edges were more abundant, and measurements could be confined within two fields from the edges (at the magnification of 500, about 0.6 mm.). In film 16R4 all the ten lymphocytes measured along the upper edge were greater than the corresponding central lymphocytes, the mean difference being $+2.35\ \mu$, therefore highly significant. Of the ten lymphocytes along the lower edge, nine were greater than the corresponding central cells, the mean difference being $+1.64\ \mu$, again highly significant. In film 16R2 the ten cells along the upper edge were all greater than the corresponding central cells, and similarly with the cells along the lower edge. The mean differences were both highly significant.

In all three films, therefore, the lymphocytes near the edges (upper and lower) were, on the average, larger than those at corresponding positions near the middle of the film.

Before any of these results are accepted at their face value, it is desirable to ask whether differences in thickness of balsam and coverglass would produce differences in magnification in the different parts of the same film, on account of slight differences in the refractive indices of objective, immersion oil, coverglass and balsam. There is apparently no definite theoretical basis for this suspicion, but a few experiments were made to test the differences in magnification of ink marks when covered with different thicknesses of glass and balsam. No significant difference was found. Moreover, the combined thickness of coverglass and balsam was determined at sixty-four equally spaced points on the principal slide, and, even if the size differences attributable to varying thicknesses of coverglass and balsam were significant, they would not account for the differences in cell size.

It may be asked: was the difference between the cell sizes due to differences in the rate of drying in different parts of the films? While the variation in size due to differences in rate of drying may be readily admitted, it must be noted that sometimes the larger cells were in thicker parts of the films, which must have taken longer to dry, while sometimes the larger cells were in thinner parts, which must have dried more quickly. Therefore the size distribution here described cannot have been due to this factor.

DISCUSSION OF UNEVEN DISTRIBUTION OF LYMPHOCYTE SIZE

The general conclusion is that in the films examined there was a real tendency for the larger lymphocytes to collect at the periphery of the films. It is true that these three films do not provide a statistical basis for the statement that this commonly occurs, but they show that it does occur, that it occurs in films that are good examples of films prepared by the slide and coverglass method, and, most important, that the phenomenon fits exactly into well established knowledge regarding the arrangement of the different leukocyte classes. Thus Gyllenswärd found that at the ends of blood films and along the edges the most peripheral leukocytes were the monocytes, the next most peripheral the eosinophils, and the next the neutrophils, while the lymphocytes were in highest proportion near the centers of the films. On the principal film of the present series several differential counts were performed and the same uneven distribution of leukocyte classes was found. It may be noted that the similarity between the peculiar lymphocyte distribution and the peculiar distribution of leukocytes in general extends to details, for Gyllenswärd found that the marginal zones (upper and lower) of peculiar leukocyte distribution extended only slightly into the film, whereas the end zone of peculiar distribution was much more extensive. Gyllenswärd emphasized the fact that the order of leukocyte distribution agreed with the commonly accepted size order of the different types of leukocytes, the largest being the monocytes and the smallest the lymphocytes. He therefore attributed the effect to the relative sizes. Silberstern confirmed the observations regarding the relative positions of lymphocytes and granular cells, but he would accept size as important only at the edges (upper and lower), where he supposed that the larger cells were stranded, when the waves of fluid receded. The abundance of granular leukocytes at the end of the film he attributed solely to surface tension phenomena, associated with differences in agglutination properties in the different leukocyte classes. His argument is very indirect and is open to several criticisms, but its chief flaw is the assumption that the crowding together of granular

leukocytes in a film is necessarily due to the same causes as the crowding together of blood cells in sedimentation tubes.

The fact that lymphocytes, cells of one class, show uneven distribution of sizes, throws considerable doubt on Silberstern's theory, and puts Gyllenswärd's suggested explanation on a sound basis. It is true that the situation is slightly complicated because (a) it cannot be assumed that the original size-ratios of the different lymphocytes are exactly represented by the size ratios of the same cells after fixation, and (b) the mass of a lymphocyte presumably does not bear a simple relation to its volume, for the larger cells have relatively more cytoplasm. These complications, however, do not invalidate the fairly simple explanation, namely that two main principles are acting. (1) The momentum of a travelling lymphocyte must be proportional to its mass, and therefore approximately to its volume, so that the larger cells will take longer to stop than do the smaller cells. (2) The mass of a lymphocyte, being approximately proportional to its volume, will be proportional to the cube of its radius, whereas the surface area will be proportional to the square of the radius. This argument is not vitiated if the shape is not quite spherical. The resistance offered by the fluid will be proportional to the surface area of the cell, and therefore the resistance will be, relative to mass (or momentum), less in the case of the larger cells.

Both of these principles will explain the accumulation of larger cells towards the end of the film, and towards the edges. The second principle will explain the more rapid fall of some large cells before the film is started, that is near the beginning, so that some large cells will be already in close contact with the slide at that time. Inertia, also greater in the larger cells, may be another factor in causing some large cells to remain near the beginning of the film.

Another mechanical explanation might be that the larger cells are carried farther because they have more difficulty in escaping from the angle between the coverglass and the slide as the blood is being drawn along. While this explanation might account for the large cells at the end of the film it would not account for the large cells at the beginning and edges.

SUMMARY

In a stained blood film prepared from a healthy male human adult by drawing the edge of a coverglass along a slide, the mean diameters (average of long axis and transverse axis) were found (magnified by approximately 1000) for all the 1658 lymphocytes, by eyepiece micrometer and oil immersion objective. The position of each cell was charted when measured. The mean of all the cell diameters was about 11 μ. Great differences between this and the means from other slides is attributed to the mode of preparation, a matter to be further investigated. The coefficient of variation, about 20 per cent, confirmed previous conclusions that size variation between lymphocytes in a film is much greater than between erythrocytes. There was a significantly greater tendency for the occurrence of unusually large lymphocytes than unusually small ones—another contrast to erythrocytes from healthy persons. There was great difference in sizes in different parts of this film, and of the other two films investigated. The sizes were greater at the upper and lower edges than at corresponding positions in the middle. The sizes near the beginning of the films were greater than those between that and the middle, and thereafter the sizes increased greatly. This can be most easily explained as due to: (a) the greater momentum of cells with greater mass, (b) the smaller resistance, in proportion to their momentum, met by large cells. This lymphocyte distribution affords a test of certain explanations offered for the disproportionate distribution of different classes of leukocytes in different parts of blood films. Gyllenswärd's explanation (size differences) is confirmed, as against Silberstern's (surface tension differences in different cell classes, associated with differences in agglutination properties).

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REFERENCES

(1) Fisher, R. A.: Statistical methods for research workers, 4th ed. London: Oliver and Boyd, 1932, pp. 307.

(2) GYLLENSWÄRD, C.: Some sources of error at differential count of white corpuscles in blood-stained smears. Acta Paediatrica, **8** suppl. **2:** 1–81. 1929.

(3) MAINLAND, DONALD, COADY, B. K., AND JOSEPH, S.: Lymphocyte sizes in human blood films. Folia Haemat., **53:** 407–425. 1935.

(4) PONDER, E.: The mammalian red cell and the properties of haemolytic systems. Berlin: Borntraeger, 1934, pp. 311.

(5) PRICE-JONES, C.: Red blood cell diameters. London and New York: Oxford, 1933, pp. 82.

(6) SILBERSTERN, E.: Untersuchungen über die Fehlergrösse bei der Differentialzählung der Leukozyten im gefärbten Ausstrich und deren in der Verteilung derselben gelegenen Ursachen. Folia Haemat., **46:** 440–474. 1932.

(7) WISEMAN, B. K.: Criteria of the age of lymphocytes in the peripheral blood. Jour. Exp. Med., **54:** 271–294. 1931.

BILIRUBIN CONCENTRATIONS IN THE HUMAN GALLBLADDER*

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The wide variation in the character of bile encountered in human gallbladders, normal in gross appearance at necropsy and operation implies a remarkably high degree of concentrative power. The bile ranges from a light yellow, almost watery fluid to a black lumpy ropy substance, having the consistence of meconium, and which can actually be cut as it streams downward from the perforated viscus. Yet it is generally accepted from the work of Hohlweg[3] and Rous and McMaster[6] that these degrees of concentration rarely exceed that of liver bile by a factor of more than ten, although the investigations of Kalk and Schöndube[5] on human bile obtained by duodenal drainage, using pituitrin as an effective cholagogue, indicate that such a factor is often exceeded in man, and may readily attain 100. Ordinary concentration factors of 40.4 and 50.9 have been demonstrated after a 24 hour fast in cats and dogs respectively by Elton and Deutsch.[2] Since casual observation suggests that the potential concentrative power of the human gallbladder has not been fully appreciated data are presented herewith to demonstrate bilirubin concentrations attained in gallbladders secured at operation and necropsy in contrast with the pigment levels found in liver bile obtained by surgical choledochostomy and by duodenal drainage.

DATA FROM GALLBLADDERS REMOVED AT OPERATION

The contents of fifteen gallbladders, exhibiting minimal pathologic changes, obtained by cholecystectomy, showed an icterus index range from 75 to 9900, and the following bilirubin levels

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in milligrams per 100 cc. by the van den Bergh method: 6.2, 6.6, 26.6, 33.3, 50, 66.6, 69.6, 71.4, 96, 125, 125, 167, 183, 305, 1000. Each gallbladder contained from 5 to 100 cc. of bile within an intact wall. The diagnosis of chronic cholecystitis was confirmed only in the first four of these specimens with the minimal bilirubin levels, the remaining eleven having walls that were grossly and histologically normal. Gallstones were present in the first and third, and the third also contained a considerable amount of pigment paste in which some cholesterol crystals could be seen. This pigment paste, occasionally encountered in gallbladders, is usually found in a relatively dilute bile, and is a probable precursor of gallstones. The phenomenon of bilirubin precipitation at a critical concentration has been found by Elton and Deutsch to be physiological in cats and dogs at the level of approximately 40 mg./100 cc., and it is quite plausible that should this same process occur in the human gallbladder it might act as the basis for calculus formation from the paste-like deposit precipitated during an arrest of the concentration process and the maintenance of the critical concentration over a sufficient interval of time. External mechanical and physicochemical factors and colloidal and crystallization forces within the paste perhaps evolve the stone or stones.

Bilirubin determinations on gallbladder bile after cholecystectomy yield important data on individual gallbladder function. It is evident in this group that definite pathology existed in all cases when the bilirubin level failed to exceed at least 50 mg./100 cc. after the usual twelve to fifteen hour fast prior to operation. Somewhat higher levels may still be indicative of functional impairment, for Caylor and Bollman,[1] who carried out similar determinations on 105 gallbladders exhibiting definite pathological changes, report a maximum of 121 mg./100 cc. Undoubtedly functional disturbances alone may impair the concentrating power of the gallbladder, as is probably true of some of the eleven "normal" cases presented herewith. These patients clinically had a typical gallbladder syndrome, hence the determination of the bilirubin content of a gallbladder after cholecystectomy gives valuable information in the absence of cholecystitis. It does not

seem unreasonable to expect concentrations to at least 200 mg./ 100 cc. within an interval of 12 hours fasting in the absence of dyskinesia or cholecystitis.

DATA FROM GALLBLADDERS REMOVED AT NECROPSY

The contents of sixty miscellaneous gallbladders, normal in gross appearance, obtained at necropsy, from patients dying under various conditions revealed a quantitative bilirubin range from 3.5 to 1786 mg./ 100 cc., and an icterus index range from 25 to 44000. These data are tabulated in table 1. The possible influences of dehydration, age, cause of death, and interval between death and necropsy were not demonstrable, and furthermore, gallbladder bile apparently undergoes a most excellent state of preservation in the intact viscus for at least 48 hours. No correlation between bile concentration and therapy or preagonal abstinence from food in prolonged illnesses could be determined, but in accidental and sudden deaths the gallbladder contents corresponded quite well with the relation to meals. Specific gravities did not consistently conform with bilirubin concentrations, although a generally rising trend with wide fluctuations from 1008 to 1059 was noted up to 908 mg/ 100 cc., followed by a decline to 1040 to 1048 in the more concentrated specimens. Reddish biles gave disproportionately high icterus indices and low bilirubin contents. Albumin was occasionally encountered. In no specimens was a greenish color, indicative of biliverdin formation, observed on opening the gallbladders. After several hours standing in open containers more or less precipitate usually formed, but the greenish change developed only in dilute or diluted specimens, associated with losses in bilirubin content up to 50 per cent in one to three days. Highly concentrated biles deteriorated remarkably slowly, many not losing their original bilirubin levels for several days. Evaporation was negligible. The various characteristics of bile from samples obtained at necropsy were comparable in every way to those of fresh specimens obtained by cholecystectomy.

TABLE 1

ANALYSES OF CONTENTS OF GALL BLADDERS REMOVED AT NECROPSY

NO.	AGE	SPECIFIC GRAVITY	BILIRUBIN CONTENT	ICTERUS INDEX	CAUSE OF DEATH
			mgm.		
1	37	1008	3.5	38.6	Septic abortion
2	7		5.0	133	Burn (albumin in bile)
3	47		5.0	25	Confluent bronchopneumonia: fibrinous pericarditis
4	56		10.0	1765	Killed in auto collision (reddish bile)
5	1		16.6	233	Empyema: multiple liver abscesses
6	44		17.5	2105	Coronary occlusion (reddish bile)
7	57		25.0	1000	Skull fracture (reddish bile)
8	0		34.0	2000	Stillborn (reddish bile)
9	54	1016	43.0	625	Killed in auto collision (reddish bile): loss to 30 mg. after 28 hrs.
10	2		53.0	1000	Burn
11	44		54.3	1133	Suicide by phenol
12	10		84.4	900	Acute nephritis
13	50	1032	85.0	1200	Intestinal obstruction
14	1		86.4	1400	Burn: negro
15	61	1025	90.0	2500	Lobar pneumonia: portal cirrhosis (reddish bile)
16	38		100.0	3530	Miliary tuberculosis (reddish bile)
17	53	1020	108.7	1222	Coronary occlusion: myocardial infarction
18	43	1021	113.6	1143	Acute appendicitis: peritonitis
19	75		120.0	2000	Perforated gastric ulcer
20	9		125.0	1250	Osteomyelitis: septicemia
21	66	1020	125.0	1250	Brain contusion: struck by auto
22	2		125.0	1667	Primary bronchopneumonia
23	1		138.0	1580	Acute enterocolitis
24	56		144.0	2444	Lobar pneumonia (reddish bile)
25	53	1018	145.0	1712	Duodenal ulcer: hemorrhage
26	5		151.0	1250	Acute appendicitis: peritonitis
27	44	1036	192.0	2418	Subarachnoid hemorrhage
28	64		200.0	3333.3	Coronary occlusion
29	72		222.0	2500	Carcinoma of prostate
30	35		236.0	2500	Postoperative hemorrhage
31	40	1025	250.0	2500	Influenzal pneumonia
32	15		250.0	2000	Acute bacterial endocarditis
33	34	1040	250.0	2105	Nephrosclerosis
34	44	1023	250.0	2944	Contusion of pancreas: fat necrosis
35	61		277.0	5000	Lobar pneumonia: portal cirrhosis
36	48	1021	278.0	2727	Contusion of cord: auto collision
37	58	1020	278.0	3333.3	Lobar pneumonia: multiple fractures

TABLE 1—*Concluded*

NO.	AGE	SPECIFIC GRAVITY	BILIRUBIN CONTENT	ICTERUS INDEX	CAUSE OF DEATH
			mgm.		
38	69		278.0	3333.3	Erysipelas
39	31		294.0	4833	Nephroslerosis
40	17		300.0	3333.3	Acute appendicitis: peritonitis
41	60	1022	316.6	2400	Subarachnoid hemorrhage: prostatectomy
42	58	1020	333.0	4300	Carcinoma of cecum: peritonitis
43	50	1033	400.0	4000	Intussusception
44	50	1028	417.0	10000	Perforated gastric ulcer
45	32		500.0	6666.6	Syphilitic meningoencephalitis
46	84	1050	500.0	30000	Pyelonephritis: diverticulum of urinary bladder
47	35	1030	500.0	21400	Acute appendicitis: peritonitis
48	46	1028	500.0	40000	Fractured skull: bronchopneumonia
49	83	1031	500.0	37500	Killed by auto
50	56	1038	500.0	10000	Brain tumor
51	20	1048	625.0	5556.0	Septic abortion
52	4		714.3	9091	Killed by auto
53	70	1059	908.0	15000	Accident: coronary stenosis
54	60	1045	1000.0	10000	Syphilitic aortitis
55	45	1042	1000.0	15384	Pyelonephritis: tuboovarian abscesses
56	18		1042.0	13333.3	Diabetes mellitus
57	21	1040	1137.0	44000	Suicide by cutthroat
58	83	1048	1786.0	17857	Coronary occlusion
59	49			1614	Acute appendicitis: peritonitis (bile contains albumin). Color incomparable after addition of alcohol
60	12			2000	Accidental asphyxia (reddish bile). Color incomparable after addition of alcohol

TECHNIC OF TESTS

Icterus index (Color density index of bile)

Undiluted bile is compared directly in the Dubosq colorimeter with a standard 1:100 solution of potassium dichromate. This standard corresponds to an icterus index unit of 100, and may be set in the colorimeter (Bausch and Lomb) at 2 to 45 mm., depending on the color density of the bile specimen. The comparison ratio multiplied by 100 gives the icterus index of the bile.

Quantitative bilirubin (van den Bergh)

All bile specimens, excepting the dilute flow obtained by duodenal drainage, must be diluted with distilled water to a titre of 1:5 to 1:100, the higher dilu-

tions being required for the higher concentrations. 1 cc. of the diluted specimen is placed in a 15 cc. graduated (centrifuge) tube and 1 cc. of Ehrlich's diazo reagent (original formula) is added. At this point a dense precipitate forms and the purple or red color of the direct reaction slowly develops. After the addition of the diazo reagent the tube is filled to the 10 cc. mark with 95 per cent alcohol to which a trace of ether has been added. The precipitate immediately disappears on shaking and a clear translucent ruby red solution of azobilirubin is obtained for colorimetric comparison with van den Bergh's cobalt sulphate standard (2 gms. anhydrous cobalt sulphate: distilled water to 100 cc.). In most specimens with adequate preliminary dilution the ruby color attains its full density within a very few minutes, but readings should be repeated at 30 minute intervals for 2 or 3 hours to obtain the maximum value. Color incomparability usually indicates the necessity for a higher dilution.

Computation: Comparison ratio times specimen dilution factor times dilution factor in centrifuge tube (10) times 0.5 equals bilirubin content of bile in milligrams per 100 cc.

DATA FROM SURGICAL DRAINAGE OF THE COMMON DUCT

In three patients bile was secured directly from the liver after cholecystectomy (two cases) or cholecystostomy (one case), and fourteen twenty-four hour specimens were available for examination.

The first patient was a white female, age 77, occasionally jaundiced. The diagnosis of extensive carcinoma of the gallbladder was made at operation and a cholecystostomy performed. During a subsequent drainage period of ten days the stools were constantly negative for bile, and the daily output of bile was noted (table 2). Twenty-four hour pigment contents varied from 24 to 157 mgm. No loss of bilirubin content occurred in any of these specimens when retested after standing for twenty-four hours in closed flasks. Specific gravities were 1011 and 1012.

The second patient was a white male, age 82, visibly deeply jaundiced for two weeks. Operation disclosed complete obstruction of the common duct by an impacted calculus and a gallbladder containing numerous small soft black stones. Cholecystectomy and common duct drainage was performed. The stools contained no bile at any time. Daily output of bile just prior to death is recorded in table 2. Specific gravity was 1007.

The third patient was a white male, age 43, with deep jaundice, clay colored stools, and complete common duct obstruction by carcinoma of the head of the pancreas. Cholecystectomy with common duct drainage was performed. Daily output of bile up to the ninth day after operation is recorded in table 2. Twenty-four hour pigment contents ranged from 23.8 to 101.4 mgm. Intravenous injection of 50 mgm. of bilirubin (Homburg) in clear alkaline solution,

although it caused a transient elevation of the serum bilirubin level from 3.7 to 5.5 mg./100 cc., did not affect the icterus index of the serum (37.5), nor was any demonstrable trace of it found in the bile, for the total pigment output following its injection was only 44.1 mgm. in 24 hours. Specific gravities ranged from 1006 to 1008.

Authentic liver bile concentrations are exhibited by these specimens, with a range from 17 to 71.4 mg./100 cc. It is reasonable to assume, however, that liver bile in health may be far more dilute than these findings indicate, since the levels found in normal gallbladders and in specimens from duodenal drainage may run as low as 3.5 and 2.0 mg./100 cc. Jones[4] has observed that in the presence of chronic cholecystitis, human liver bile tends to be

TABLE 2

DAILY ANALYSES OF BILE OBTAINED BY SURGICAL DRAINAGE

DAY	CASE 1		CASE 2		CASE 3	
	Amount	Bilirubin	Amount	Bilirubin	Amount	Bilirubin
	cc.	*mg./100 cc.*	*cc.*	*mg./100 cc.*	*cc.*	*mg./100 cc*
1	120	20.0	85	30.0	310	17.8
2	221	71.4	55	30.0	325	31.2
3	265	33.3			150	28.0
4	300	34.4			140	17.0
5	250	25.0			160	38.5
6					310	25.0
7					212	20.8

more concentrated than normal, and a similar phenomenon has been noted in cats and dogs by Elton and Deutsch* after cholecystectomy, when the liver flow is collected in a thin rubber sac placed within the abdominal cavity, long after the possible effect of ether anesthesia has ceased to operate. This increase in the concentration of liver bile following impairment of gallbladder function may be attributed to a natural compensatory decrease in the water output of the liver as a check on choleresis, and variations in the pigment content of liver bile apparently depend on this regulation of the water output. Choleresis appears quite analogous to diuresis. That choleresis has little to do with pig-

* Unpublished data.

ment output is attested by the fact that the pigment content of a copious flow of conspicuously bright yellow bile may be almost negligible as contrasted with that of a few cubic centimeters of a darker and less conspicuous flow.

DATA FROM DUODENAL DRAINAGE

Biliary drainage with the Rehfus tube and olive oil was performed on nineteen patients. Olive oil usually acts as an effective choleretic agent, although its cholagogic action is but slight, and it has the great advantage of its relative immiscibility with the duodenal contents. Seven of these drainages were conducted by a fractional method, consisting simply of changing the receiving bottles at intervals of 15 to 20 minutes in order to detect all changes in concentration during a period of at least three hours after the beginning of the flow. By this method the last few specimens reveal whether or not dilution occurs after prolonged choleresis. Sufficiently conclusive data are not yet at hand properly to evaluate the factor of choleresis in relation to gallbladder dysfunction, but the results to date have been very consistent. With the normal gallbladder there occurs at first a slight cholagogic response to the contact of the olive oil with the duodenal mucosa, and traces of gallbladder bile come through (25–80 mg./100 cc.), then progressive dilution takes place as the liver flow is established, finally yielding bile averaging 8 to 10 mg./100 cc. in bilirubin content, and not infrequently dropping as low as 2.0 mg./100 cc. in the final clear yellow, slightly viscous alkaline specimen of specific gravity 1006 to 1008. In the presence of chronic cholecystitis or dyskinesia the fractions fail to exhibit such dilution, and the bilirubin level remains well above 10 mg./100 cc. throughout the three hour interval.

SUMMARY

The bilirubin contents of fifteen selected gallbladders removed surgically and exhibiting minimal pathologic changes (eleven anatomically normal) ranged from 6.2 to 1000 mg./100 cc. All concentrations below 50 mg./100 cc. were found in the four specimens showing definite chronic inflammatory reactions, and are

not acceptable as satisfactory minima because of possible dilution in a pathological gallbladder. The contents of sixty apparently normal gallbladders obtained at necropsy exhibited bilirubin levels of 3.5 to 1786 mg./100 cc, from which a concentration factor of 510 may be computed. Liver bile obtained by surgical choledochostomy in three patients with complete common duct occlusion ranged from 17 to 71.4 mg./100 cc. in fourteen twenty-four hour specimens. The usual bilirubin content of liver bile obtained by duodenal drainage in individuals with no known gallbladder disease is 8 to 10 mg./100 cc., with a further possible decrease to 2.0 mg./100 cc. after prolonged choleresis.

The great variability in the bilirubin content of liver bile, ranging from 2.0 to 71.4 mg./100 cc., due on the one hand to the increased water output of the liver after prolonged choleresis in individuals without gallbladder dysfunction, and on the other hand to the inhibition of choleresis in cholecystic disease and hepatic injury, makes it difficult to select a satisfactory average minimum level in liver bile for comparison with the bilirubin contents of gallbladders. However, a concentration factor of 105 may be computed from the fact that liver bile obtained by surgical drainage contained 17 mg./100 cc. and that a normal gallbladder contained 1786 mg./100 cc. A factor of 125 to 100 may be computed from the observation that liver bile in the absence of cholecystic disease ranges 8 to 10 mg./100 cc. and that it is not unusual for a gallbladder to contain bile of 1000 mg./100 cc. Since it is possible for a clear alkaline liver bile to have a bilirubin level as low as 2.0 mg./100 cc., an extreme combined factor for liver and gallbladder jointly might be computed as 893. A most conservative concentration factor, attained without undue fasting, appears to be from 30 to 50.

REFERENCES

(1) Caylor, H. D., and Bollman, J. L.: The bilirubin content of gallbladder bile in cholecystic disease. Arch. Path. and Lab. Med., **3:** 993–1001. 1927.

(2) Elton, N. W., and Deutsch, E.: Concentration and precipitation of bilirubin in the gallbladder and bile ducts; experimental studies on dogs and cats. Arch. Path., **15:** 818–827. 1933.

(3) HOHLWEG, H.: Ueber Störungen der Salzsäureabscheidung des Magens bei Erkrankungen und nach Exstirpation der Gallenblase. Deutsch. Arch. f. klin. Med., **108:** 255–276. 1912.

(4) JONES, C. M.: Blood pigment metabolism and its relation to liver function. Arch. Int. Med., **29:** 643–668. 1922.

(5) KALK, H., AND SCHÖNDUBE, W.: Über moderne Methoden in der Diagnostik der Gallenblasenerkraukungen. Med. Klin., **21:** 1949–1952. 1925.

(6) ROUS, P., AND MCMASTER, P. D.: The concentrating activity of the gall-bladder. Jour. Exper. Med., **34:** 47–73. 1921.

EDITORIAL

The Thick Smear Method of Diagnosing Malaria

As more and more commercial contacts are established between the temperate zone and the tropics and subtropics, one may expect to encounter the diseases of these warmer climates in the northern parts of the United States, and in Canada. The methods now employed in obtaining fruits from the tropics and southern parts of the United States and the rapid means of transportation provided for man, animals and native foods, make the appearance of certain tropical diseases, especially malaria, of increasing importance to those who examine patients in the north temperate zone.

In most cases, the detection of plasmodia in ordinary thin smears of blood when the patient is in the throes of an acute attack of malaria is not attended with much difficulty. But in old burned out malaria or in chronic, mild or quiescent infections, this is another matter and requires some modification of procedures. Even in some acute, and especially early typical cases, the parasites may be scarce and much time may be wasted in searching a slide; this is especially true in early stages of malaria. Under such circumstances, even if an occasional parasite is found, its species may be in question until, after further search, other forms are seen. These difficulties were clearly recognized by the first investigators searching for the then but newly recognized cause of malaria. Since that time several methods have been devised, including the administration of epinephrine to drive out the parasites entrapped in the spleen, the many attempts to cultivate the parasite in vitro, and the concentration method of Bass and Johns. This last method has been used to advantage in certain laboratories but requires too much manipulation and equipment to make it popular in a field survey or for use in State Board of Health Laboratories. The method requires the taking

of 10 cc. of blood in a special anticoagulating fluid, and by alternately centrifuging and skimming off the top layers three times, the concentration is accomplished. The procedure requires about half an hour before the slide is ready for staining. The authors claim it concentrates the parasites 900 times, and its chief advantage seems to be in cases of estivo-autumnal malaria; when crescents of *Plasmodium falciparum* are scarce, the rings of *Plasmodium vivax* and *Plasmodium malariae* do not concentrate so much. It is interesting to note that in 1903 Ross indicated he had attempted concentration by centrifuging dehemoglobinized blood, but the details of the method were not given.

As far back as 1895, Ross began to use thick smears for diagnosing malaria and he described the method in a lecture delivered in 1902. The method was adopted from Sir Patrick Manson's method of detecting filaria and "flagellate bodies" in blood. Since then, it has been modified by numerous investigators, notably, Ruge, Barber and Komp, and James. While the details of the method may be had by consulting the numerous papers on the subject, it is important to note the great stress laid on the cleanliness of the slides, the fact that several drops of blood should be used, and that the area covered by the blood should be not greater than about 1 cm. in diameter. After thorough drying, the blood may be dehemoglobinized by distilled water, acetic acid, or, as the late Fülleborn suggested, the slide may be stained in an aqueous solution of stain which will lake the erythrocytes during the staining process. The stain of choice is Giemsa's, and a special method of preparing it without the use of azure II has been described by Komp.

All investigators have indicated by various references that the most disappointing accident that may happen in this method is the failure to have the smear stick to the slide. They imply that this catastrophe is caused by the use of dirty slides, contact of the slide with the patient's skin, or staining before thorough drying. Several methods have been proposed for actually making the smear but no one implies that this phase of the procedure is critical. Thus, some use a toothpick; others, like Krauss, "with the corner of another slide spread" the drops of blood "to the size

of a one-cent piece by describing centrifugal circles"; still others use the side of the needle to make the smear. As a matter of fact, this is the critical stage in the process, and unless the blood can be made to adhere to the slide there is no value in the method. It is best to stir the blood well with whatever instrument is used so that the area of the glass under the drop is thoroughly wet with the blood. Krauss' method is excellent for this purpose and, if energetically carried out, will help to throw the infected cells to the periphery where the area for searching is limited.

Just what factors are involved in causing the blood to stick to the slide are unknown but it would appear that thorough wetting of the glass surface with the blood is essential. It may be that the stirring partially defibrinates the blood and this may be a factor since defibrinated blood does stick well to the slide. In other words, stirring may break up the long threads of fibrin and thus the blood is permitted to dry rather than clot. If long threads of fibrin form, their contraction may be the cause of the smear loosening from the slide. Whatever the factors are, the process of stirring accomplishes the result.

The degree of concentration of the parasites is not clearly defined. Ross indicated the concentration was of the order of about twenty-five times that of ordinary liquid films used about the early part of the twentieth century. Bass and Johns thought the thick smear was ten times more concentrated than the thin ones. James considered the concentration fifty times and believed the parasites may be readily found when present in as low a number as 1 in 100,000 cells. He further believed this method to be one of the most accurate of all laboratory procedures, and said that if two thick smears taken at suitable intervals were competently examined and found to be negative, malaria could be excluded from diagnostic consideration. Sinton stated that one could find *Plasmodium vivax* in thick smears in less than a sixth the time required for finding them in thin smears, and it would take a twenty-third the time to find *Plasmodium falciparum*.

While there is some difference of opinion as to how long one should examine a thick smear before considering it negative, it would appear, from information available, that a slide may be

considered negative when from 50 to 150 fields (oil immersion), carefully examined, reveal no parasites; this should take an experienced observer from three to seven minutes. The recognition of the species of Plasmodium is perhaps more difficult in thick smears than it is in thin smears, but the larger number that may be found in thick smears gives some advantage in this respect.

For those who encounter malaria only occasionally and then not in the acute, typical forms, the thick smear adds a distinct addition to diagnostic methods, and in field surveys and State Board of Health Laboratories, the method will save much time and will bring about more accurate results. In giving instructions for making the smears, it is important that emphasis be placed on rubbing or stirring the drops of blood thoroughly over the restricted area on the slide in order to assure the film sticking during the staining process.

—T. B. MAGATH

BOOK REVIEWS

The Bacteriology of Typhoid, Salmonella, and Dysentery. By Leon C. Havens. New York: The Commonwealth Fund, pp. v + 158, 1935. $1.75.

This important monograph is the first to appear since Nichols' book on Carriers published in 1922. The book consists of thirteen chapters dealing with the general problem including the antigenic composition of the bacterial cell, bacterial variation, culture media, the preservation and isolation of bacterial pathogens in fecal specimens, and their identification by cultural and serologic methods. Then follow chapters dealing specifically with typhoid fever and the carrier problem, the Salmonella group and their importance in food poisoning and finally the dysentery group.

Throughout the book, the author emphasizes the importance of repeated cultures of feces in order to make proper diagnoses, bringing out the oft neglected fact that agglutination tests in these diseases are of relatively little importance as compared with actual isolation of the organisms. This is particularly true in searching for carriers where the Widal test has no bearing on the problem, according to Havens. In the author's experience, ten per cent of typhoid patients become carriers while a less number of persons become carriers who have bacillary dysentery.

He lists and discusses six Salmonella pathogens for man. Several chapters give details of the various bacterial procedures in the study of these diseases and particularly important are the instructions for preserving stools for future culture.

Bibliographic references are plentiful and the book has a most pleasing appearance. It will be an important addition to all bacteriology laboratories.

Textbook of Bacteriology. By Thurman B. Rice. Philadelphia and London: W. B. Saunders Company, pp. 551, 1935. $5.00.

This is an elementary text designed for first year students in

bacteriology and the author has succeeded well in reducing the amount of unnecessary material which usually is found in texts of bacteriology. The style is direct and brief, covering all of the essential things that should be taught in a first year course. Long and theoretical discussions concerning controversial subjects are eliminated and the more practical phases of the subject are dealt with. The technical procedures given in the book are those which may be carried out by physicians having a limited amount of equipment.

Diet and Die. By Carl Malmberg. New York: Hillman-Curl, Inc., pp. 149, 1935. $1.50.

This excellent exposé of fads and fadists who have plied their trade on the unsuspecting public is well worth reading. Physicians can do a real service for their patients by seeing that they procure a copy of it.

NEWS AND NOTICES

Fifteenth Annual Convention of the American Society of Clinical Pathologists

The Fifteenth Annual Convention of the Society will be held in Kansas City, Missouri, May 8 to 10. The present plans indicate that the first two days will be devoted to a general program, Friday night to a round table discussion, and Saturday night to the annual banquet, the business meeting being held on Sunday night. Those wishing to appear on the program are urged to send their titles of papers and scientific exhibits to the Secretary not later than March 1.

Just preceding the annual meeting, on May 6 and 7, the Society will conduct a two-day seminar. One day will be devoted to Hematology under the direction of Dr. Hal Downey and the second day will be devoted to the study of tumors under the direction of Dr. R. L. Jaffé. Anyone planning to attend these seminars is requested to send his name as soon as possible to the Secretary.

Dr. Lawrence Weld Smith has been appointed Professor of Pathology to the chair left vacant by the death of Professor John I. Fanz, at Temple University, School of Medicine.

Dr. Ernest E. Aegerter has been appointed instructor in pathology in the same department.